Oligopeptide-functionalized Graft Copolymers: Synthesis and Applications in Nucleic Acid Delivery

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OLIGOPEPTIDE-FUNCTIONALIZED GRAFT COPOLYMERS: SYNTHESIS AND APPLICATIONS IN NUCLEIC ACID DELIVERY

A Dissertation Presented

by

REBECCA BOUDREAUX BREITENKAMP

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2009

Polymer Science and Engineering
OLIGOPEPTIDE-FUNCTIONALIZED GRAFT COPOLYMERS: SYNTHESIS AND APPLICATIONS IN NUCLEIC ACID DELIVERY

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REBECCA BOUDREAUX BREITENKAMP

Approved as to style and content by:

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ACKNOWLEDGMENTS

I would like to thank my advisor Professor Todd Emrick. I appreciate your willingness to take a risk on a third year graduate student and enabling me to explore my research interests in nucleic acid therapy. I am grateful for your guidance, patience, and persistence throughout my Ph.D. work. Thank you to Professor Murugappan Muthukumar and Professor Craig Martin for their willingness to serve on my thesis committee and for providing insight throughout this process. The members of the UMass Polymer Science and Engineering Department faculty and staff are greatly appreciated for the contributions to my education and research.

Thank you to the MURI Department of Defense grant, the University of Massachusetts School of Management Isenberg Scholar Program, the Delta Delta Delta Foundation, and the NSF-sponsored MRSEC on Polymers (IRG III) for research funding.

Much appreciation is owed to the past and present members of the Emrick Research Group. In particular, I would like to thank Dr. Bryan Parrish for collaborating with me on the oligopeptide-functionalization of polyesters. Thank you to current members Katrina Kratz and Brent Hammer for making my second hiatus at UMass entertaining.

I would like to thank Dr. Yemin Liu, Professor Theresa Reineke, Jeff Kane, Dr. Sangram Parelkar, Kim Wojeck, Professor Larry Schwartz, and the Schwartz Research Group for helping a synthetic chemist learn cellular and molecular biology techniques and allowing me to use their equipment and instrumentation. I would like to acknowledge Dr. Zhaoyang Ou, an outstanding collaborator, for his work on characterizing the oligopeptide-functionalized polyolefins. I appreciate the help from
Professor David Hoagland, John Harner, and Alexei Popov with gel electrophoresis and aqueous GPC. Thank you to Jack Peters, Roberto Laos, and Dr. Ticora Jones for being fun fellow group members in my early years of graduate research.

Professors Michael Malone, Soren Bisgaard, and Joseph Stokes deserve a special thank you for opening my eyes to the world of business, helping me take an idea on paper and turning it into a viable start-up company, and the on-going mentoring that you have provided.

Thank you to Barry Suprenant, Yung Morgan, Doug Holmes, Meghan Geraldi Holmes, and Donna Wrublewski for their friendships and making my time in Conte and Amherst more fun. Thank you to Dr. Upma Sharma for being a wonderful friend and keeping me motivated to finish graduate school.

To my family – Mom, Dad, Ryan, Erik, Lyndi, Sean, and Ally – thank you for your love, support, and believing in me. I appreciate you always being there. To my grandmother, Ella Ruth Blanchard, I appreciate your friendship, love, and encouragement throughout my education.

Most importantly, I would like to thank my husband and best friend Chip for being a wonderful partner, friend, and colleague. In addition to being supportive throughout the graduate school journey, he served as an excellent scientific and business collaborator in both my academic and start-up endeavors. I cannot wait to see where the next adventure takes us.
ABSTRACT

OLIGOPEPTIDE-FUNCTIONALIZED GRAFT COPOLYMERS: SYNTHESIS AND APPLICATIONS IN NUCLEIC ACID DELIVERY

FEBRUARY 2009

REBECCA BOUDREAUX BREITENKAMP, B.S., UNIVERSITY OF SOUTHERN MISSISSIPPI

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Todd S. Emrick

Utilizing the diverse functionality of amino acids, a new class of amphiphilic graft copolymers has been synthesized, characterized, and explored for applications in biomaterials and nucleic acid delivery. This thesis research focused on the syntheses of oligopeptide-functionalized polyesters and polyolefins. Polyester functionalization was geared towards applications in biomaterials, tissue engineering, and drug delivery by incorporating sequences that promote cell-adhesion. These polyester-\textit{graft}-oligopeptide materials were prepared by a 1,3-Huisgen cycloaddition reaction, “click” chemistry, of an azide-terminated oligopeptide (prepared by Fmoc-based solid phase peptide synthesis (SPPS)) and alkyne-containing polyester (synthesized by ring-opening polymerization). Following the syntheses of these materials, they were analyzed by nuclear magnetic resonance (NMR) and organic gel permeation chromatography (GPC).

The oligopeptide-functionalized polyolefins were designed for nucleic acid complexation, and therefore the oligopeptide sequences were intended to incorporate positively-charged moieties (\textit{e.g.}, oligolysine) for DNA and short interfering RNA (siRNA) complexation. These \textit{graft} copolymers, prepared by SPPS followed by ring-
opening metathesis polymerization, have highly tunable structures that enable control over charge density and polymer backbone rigidity. Moreover, non-ionic hydrophilic grafts such as polyethylene glycol were integrated into these polyelectrolytes such that the charges along the polymer backbone are spaced accordingly while maintaining the hydrophilicity of the polymer. While numerous applications for such charged, “bio-tailored” materials can be envisioned, this work is geared towards positively-charged polyelectrolytes for their potential application in nucleic acid therapy, specifically the delivery of plasmid DNA and siRNA. These graft copolymers were characterized (\(^1\)H, \(^{13}\)C NMR, organic and aqueous GPC), studied for their solution properties (static and dynamic light scattering), and investigated as polyplexes with plasmid DNA.
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<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>A₂</td>
<td>second viral coefficient</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>ADMET</td>
<td>acyclic diene metathesis</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Cell Culture</td>
</tr>
<tr>
<td>ATR</td>
<td>attenuated total reflectance</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbamate</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
</tr>
<tr>
<td>But</td>
<td>butyl</td>
</tr>
<tr>
<td>C</td>
<td>Celcius</td>
</tr>
<tr>
<td>CHN</td>
<td>carbon hydrogen nitrogen</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>COS-1</td>
<td><em>Cercopithecus aethiops</em> African green monkey kidney cells</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole or 2-(4-amidinophenyl)-1H-indole-6-carboxamidine</td>
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<tr>
<td>D(c)</td>
<td>diffusion coefficient</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<tr>
<td>DIC</td>
<td>diisopropylcarbodiimide</td>
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<tr>
<td>DIPEA</td>
<td>diisopropylethylamine</td>
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<td>DLS</td>
<td>dynamic light scattering</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dn/dc</td>
<td>change in refractive index/change in concentration</td>
</tr>
<tr>
<td>D₀</td>
<td>diffusion coefficient at zero concentration</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
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<td>EI</td>
<td>electron ionization</td>
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<td>eq</td>
<td>equivalent</td>
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<td>EtOAc</td>
<td>ethyl acetate</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FTIR</td>
<td>Fourier Transform infrared</td>
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<td>9-fluorenylmethoxycarbonyl</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>g²(t)</td>
<td>scattering intensity autocorrelation</td>
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<td>Gly</td>
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<td>GPC</td>
<td>gel permeation chromatography</td>
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<td>GRGDS</td>
<td>glycine-arginine-glycine-aspartic acid-serine</td>
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<td>1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide</td>
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<td>O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate</td>
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<tr>
<td>HeLa</td>
<td>human adenocarcinoma epithelial cervical cells</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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HOBT 1-hydroxybenzotriazole
HRMS high-resolution mass spectrometry
HRMS-FAB high-resolution mass spectrometry fast atom bombardment

\( k_B \) Boltzmann constant

L chain contour length

Leu leucine

\( l_p \) persistence length

l Ile isoleucine

IR infrared

M mole/Liter

\( m \) multiplet

MeOH methanol

MHz megahertz

min minute

\( \mu L \) microliter

mL milliliter

\( \mu m \) micrometer

mm millimeter

mmol millimole

\( M_n \) number-average molecular weight

mol mole

\( M_p \) peak molecular weight

MRI magnetic resonance imaging
<table>
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<tr>
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<tr>
<td>( M_w )</td>
<td>weight-average molecular weight</td>
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<tr>
<td>MWCO</td>
<td>molecular weight cutoff</td>
</tr>
<tr>
<td>( \eta )</td>
<td>solvent viscosity</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>N:P</td>
<td>ratio of protonatable nitrogens in the polymer (N) to DNA phosphates (P)</td>
</tr>
<tr>
<td>PAMAM</td>
<td>polyamidoamine</td>
</tr>
<tr>
<td>Pbf</td>
<td>2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl</td>
</tr>
<tr>
<td>PDI</td>
<td>polydispersity index</td>
</tr>
<tr>
<td>pDNA</td>
<td>plasmid DNA</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
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<td>poly(ethylene imine)</td>
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<td>( R_g )</td>
<td>radius of gyration</td>
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</table>
RGD  arginine-glycine-aspartic acid
R_h  hydrodynamic radius
RI   refractive index
RNA  ribonucleic acid
RNAi ribonucleic acid interference
ROMP ring-opening metathesis polymerization
s    singlet
SDGRG serine-aspartic acid-glycine-arginine-glycine
siRNA short interfering ribonucleic acid
SLS  static light scattering
SPPS solid phase peptide synthesis
sxt  sextet
T    temperature
t    triplet
TAE  tris-acetate-EDTA
TFA  trifluoroacetic acid
TFE  trifluoroethanol
THF  tetrahydrofuran
TIPS triisopropylsilane
UV   ultraviolet
Val  valine
wt   weight
CHAPTER 1

INTRODUCTION

1.1 Introduction

Polymer materials are attractive candidates for many applications due to their unique properties and tunability in terms of structure and function. Synthetic polymers can be tailored for biological applications by controlling functionality, molecular weight, degradation characteristics, and physical properties (Figure 1-1). Recognizing the value of synthetic polymers as therapeutics and drug delivery vehicles, Ringsdorf and coworkers pioneered the idea of polymer-based therapeutics over 30 years ago. Since this time, this field has expanded to include such technology platforms as polymer drugs, polymer-drug conjugates, polymer micelles, and ionic multicomponent polyplexes. In efforts to prepare polymers for specific areas such as biomaterials, tissue engineering, and drug delivery, biologically-relevant moieties such as oligopeptides, sugars, viruses, and drugs are often introduced as functional initiators, end groups, or pendent side chains to enhance biocompatibility and water solubility and enable targeted delivery for therapeutic applications (Figure 1-1). The incorporation of therapeutic moieties in polymers presents synthetic and characterization challenges as the physical properties of such conjugates are often atypical of traditional polymer materials, requiring the use and development of techniques specific for each type of therapeutic reagent or conjugate.

To date, linear polymers have been most utilized in polymer therapeutics due to the relative ease of synthesis and characterization, ability to synthesize materials with low polydispersity by controlled polymerization techniques, and the wide range of degradable
and non-degradable polymer backbones available. In addition to linear polymers, branched polymers, graft or comb, and dendritic polymers can be used, and in many cases are degradable. Despite the synthetic complexity associated with preparing branched and dendritic polymers, they offer attractive attributes for polymer therapeutics.

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<table>
<thead>
<tr>
<th>Polymer Architectures</th>
<th>Advantages of Polymers</th>
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<tr>
<td>linear polymers</td>
<td>• Relative ease of synthesis</td>
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<td>branched polymers</td>
<td>• Scalability</td>
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<td>dendrimers</td>
<td>• Tunable parameters such as</td>
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<td>graft copolymers</td>
<td>- Molecular weight</td>
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<td>- Degradation rate</td>
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<td></td>
<td>- Mechanical properties</td>
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<td>- Functionality incorporation</td>
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**Technology Platforms**

<table>
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<tr>
<th>Polymer-drug conjugates</th>
<th>Biologically-relevant Moieties</th>
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<tr>
<td>drug-loaded polymer micelles</td>
<td>folate small-molecule targeting group</td>
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<tr>
<td>polypexes</td>
<td>doxorubicin cancer therapeutic</td>
</tr>
<tr>
<td></td>
<td>RGD sequence oligopeptide targeting group</td>
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</table>

**Figure 1-1. Examples of polymer architecture and conjugation for therapeutics**

2
including the ability to introduce extensive and diverse functionality onto a single polymer chain.

To exploit the unique attributes of polymer architecture, branched structures have been prepared and studied as potential polymer-based therapeutics. For example, Stiriba, Kautz, and Frey examined hyperbranched and linear polyglycerols, which have potential for therapeutic delivery applications as nanocapsules. Unlike their linear analogs, hyperbranched polyglycerols were able to encapsulate hydrophilic, small molecule dyes, illustrating the benefit of macromolecular architecture for encapsulation/delivery applications.

Dendritic macromolecules are perfectly-branched structures that have been designed recently for in vivo applications such as drug delivery and magnetic resonance imaging (MRI) contrast agents. During the early 1990’s, Newkome et al. synthesized unimolecular micelles consisting of an amphiphilic dendrimer with a hydrophobic interior and hydrophilic periphery. Due to the interior hydrophobic cavity, these materials offered the potential to encapsulate hydrophobic, small-molecule drugs. Fréchet and coworkers have also evaluated a variety of dendrimers and dendritic-like macromolecules for applications in drug delivery. Taking advantage of the unique structure and properties of dendrimers, degradable polyester-based systems, pH-responsive assemblies, dendrimer-drug conjugates, small molecule and protein drug delivery agents, and targeted delivery systems have all been synthesized based on these three-dimensional branched structures.

The use of dendrimers as MRI contrast agents has also been explored, beginning with the work of Wiener, Tomalia, and coworkers. These contrast agents consist of
amine-functionalized PAMAM dendrimers conjugated to chelator moieties, enabling the binding of gadolinium ions. The ability to attach numerous chelating moieties to the dendrimer periphery is advantageous for imaging. Some dendrimer-metal chelate conjugates bind up to 170 gadolinium ions, far exceeding the degree of functionalization possible in linear polymer systems, where steric crowding limits the extent of chelation possible.

While dendrimers offer promise for a variety of therapeutics, questions concerning their cytotoxicity and scalability due to their multi-step synthesis have to date limited their commercialization. In this thesis work, efforts are made to integrate the advantages of branching through graft copolymer architectures, prepared by simple, conventional polymerization methods. Specific graft copolymer targets include aliphatic polyesters, which are hydrolytically degradable, and hydrophilic polyolefins, which are hydrolytically stable. Both types of materials carry potential in therapeutic applications, which will be introduced below and described specifically in subsequent chapters.

1.2 Polyesters for Therapeutics

The synthesis of polymers containing biologically active groups has been undertaken with a variety of polymer structures including polyesters, polyanhydrides, polyethers, and polymethyl(acrylates) as the backbone structure. Langer and coworkers have studied degradable polymers as scaffolds for polymer-drug conjugates, delivery of protein and DNA drugs, targeted therapeutics, and tissue engineering. Recently, polymer-drug conjugates were prepared from commercially available dextran, a biodegradable, biocompatible material, by its covalent conjugation to the chemotherapy drug methotrexate. The polymer and drug were linked by an enzyme-specific
oligopeptide sequence, Pro-Val-Gly-Leu-Ile-Gly, which is cleaved selectively by matrix metalloproteinases, zinc-dependent enzymes found in elevated levels in cancerous tissue. This design enables the site-specific enzymatic cleavage and release of the active drug. These conjugates showed promise as an improved treatment method by offering increased solubility and stability over the free drug and are currently under investigation to determine in vivo stability and efficacy.

Other efforts in polymer therapeutics center on the development of biocompatible polymer materials for tissue engineering applications. These systems utilize degradable polymers that often incorporate oligopeptide sequences and hydrophilic groups provide biocompatibility, water solubility, and receptor-ligand interactions. For example, Hubbell and coworkers synthesized a cross-linked poly(ethylene glycol)-co-poly(acrylic acid) network that was functionalized subsequently with RGD-containing peptide sequences through the carboxylic acids in the poly(acrylic acid) block to promote polymer-cell adhesion. In addition, Langer and coworkers have reported the pendent functionalization of poly(lactic acid-co-lysine) with the adhesion-promoting sequence RGD for material applications where cell growth is desired (e.g., tissue engineering). This was accomplished by ring-opening polymerization of a lactone with a protected lysine side chain, which was then deprotected to give free primary amines. The acid terminus of the RGD-containing oligopeptide was coupled to this amine on the polyester to generate pendent, oligopeptide functionality. Despite the progress this work exemplified in the incorporation of oligopeptides into a hydrolytically degradable system, problems encountered using this methodology included crosslinking, low peptide incorporation (less than 1 mole percent), and polyester backbone degradation. Such
issues underscore the need for compatibility between the backbone structure and
tool functionalization conditions.

1.3 Polyolefins in Biomaterial Applications

Polyolefins such as polyethylene and polypropylene are classic commercial
plastics not often viewed as biologically relevant. However, polyolefins have been
explored in biological applications with their hydrolytically stable properties making
them suitable as implants and structured materials.\textsuperscript{42-44} Due to its functional group
tolerance,\textsuperscript{45,46} modern metathesis chemistry has enabled the synthesis of wide variety of
polyolefins including those with pendent carboxylate,\textsuperscript{47} ammonium,\textsuperscript{48} and oligopeptide\textsuperscript{49-}
51 groups. For example, as shown in Figure 1-2, acyclic diene metathesis (ADMET) has
been used to incorporate single amino acids, peptide dimers, or trimers into unsaturated
hydrocarbon polymer backbones. Hopkins and Wagener described the integration of
various amino acids into these polyolefins using acyclic diene monomers and Grubbs’
Generation II catalyst. The resulting semi-crystalline material exhibited changes in its
melting temperature due to the incorporation of these amino acid moieties.\textsuperscript{49,52-54}

Using ring-opening metathesis polymerization (ROMP), Grubbs, \textit{et al.} prepared
polyethers with phenylalanine groups\textsuperscript{55} and poly(norbornene)s with bioactive moieties
such as GRGDS and PHSRN to synergistically inhibit cell binding to fibronectin-coated
surfaces to demonstrate the bioactivity of these oligopeptide-functionalized polymers.\textsuperscript{50,51}

In the polynorbornene examples (Figure 1-2), a variety of monomers were prepared and
functionalized with individual amino acids or oligopeptide sequences and then
polymerized using Ru-based ROMP. Homopolymers of these monomers were prepared
as well as copolymers with short PEG chains. The resulting GRGDS and PHSRN
materials were found to substantially reduce cell adhesion to fibronectin-coated surfaces due to the ligand-based polymer-cell interactions, demonstrating the promise of ROMP-derived materials for biological applications.

Figure 1-2. Application of metathesis techniques to synthesize bio-tailored polyolefins

ROMP has also been used to incorporate other biomolecules, such as sugars, into polyolefins. Grubbs and Fraser reported well-controlled, low polydispersity polynorbornenes with two sugar groups per repeat unit, illustrating the functional group tolerance of these ruthenium catalysts and enabling the synthesis of polyolefins with highly-functional side chains.\textsuperscript{22} Kiessling and coworkers have also prepared saccharide-containing polynorbornenes to prepare polyvalent scaffolds capable of protein recognition.\textsuperscript{21,23} These polymers demonstrated both binding efficiency and recognition specificity in their ligand interactions with the mannose/glucose-binding protein concanavalin A, resulting in their potent inhibition of cell agglutination (\textit{i.e.}, the aggregation of cells as a result of the protein agglutinin on the cell surface). The multivalent nature of these polymers provides superior activity relative to the monovalent
sugar derivatives. One polynorbornene-mannose derivative exhibited a 50,000-fold increase in inhibitory activity.\textsuperscript{23}

\section*{1.4 Polymers for Nucleic Acid Delivery}

Synthetic, cationic polymers have emerged as reagents for complexation of plasmid DNA (pDNA) and short interfering RNA (siRNA), termed polyplexes. Because of the potential beneficial, medical impact of effective nucleic acid delivery, numerous research labs have pursued this topic over the past 15-20 years.\textsuperscript{21} Science magazine’s scientific breakthrough of 2002 was the RNA interference (RNAi) process, promoting these small RNAs as having the potential to revolutionize modern medicine.\textsuperscript{56} RNAi is the process by which short, single-stranded RNA (20-22 bases in length) binds messenger RNA (mRNA) in the cytoplasm, marking the mRNA for destruction and therefore preventing its protein expression. This process can be used to knockdown specific genes to better determine their role or function in the cell. On the therapeutic side, small RNAs, also known as short interfering RNA (siRNA), have been shown to shut down diseased cells for cancer and HIV \textit{in vitro}.\textsuperscript{57-59} Shortly after their discovery of the RNAi process, Mello and Fire were awarded the 2006 Nobel Prize in Medicine. However, despite the magnitude of the accomplishment, practical therapeutic applications for siRNA have been slowed by ineffective delivery methods. Improving the delivery of these short nucleic acids will enable progress toward clinical use of RNA treatments for cancer, HIV, and other diseases. Indeed delivery is at the core of siRNA therapy and other nucleic acid treatments, making it imperative the development of new materials that can safely deliver these reagents to the desired \textit{in vivo} target(s).
An ideal nucleic acid delivery system would encapsulate the desired nucleic acid, deliver it only to diseased cells, and efficiently express of the encapsulated nucleic acid (in plasmid DNA therapy) or shutdown the complementary mRNA (in siRNA therapy) while remaining non-immunogenic and non-cytotoxic throughout the body. While the goal of gene therapy is to transport the genetic material to the nucleus where it can be expressed, siRNA therapy focuses on the shutdown of diseased genes. The goal of delivering siRNA is to transfect this 20-22 base pair oligonucleotide sequence to the cell’s cytoplasm, where it will bind with complimentary mRNA, marking it for destruction and preventing the mRNA from producing the undesired proteins associated with the disease.\textsuperscript{60,61}

![Figure 1-3. Ideal nucleic acid delivery system](image)

Currently, the primary approach to nucleic acid condensation and delivery uses viral vectors. Genetically-modified viruses, such as adenoviruses and retroviruses, provide excellent transfection efficiencies and subsequent gene expression. However, serious safety issues, including the onset of leukemia-like symptoms and patient death in clinical trials,\textsuperscript{62,63} have called into question the clinical viability of this approach. Non-viral approaches to gene therapy are gaining momentum especially using cationic liposomes, small molecule and polymer-DNA conjugates, and polymer micelles for the
condensation or packaging of genetic material. Despite their reduced transfection abilities, non-viral systems exhibit fewer safety issues and can be better tailored for the desired application. Synthetic polymers, in particular, offer modularity in synthetic design with the ability to control molecular weight, polymer backbone, degradation abilities, and incorporation of targeting moieties and other functionalities.

The complexation of DNA with cationic materials such as polylysine and polyarginine have been studied for over 40 years. In 1995, Boussif and coworkers first introduced poly(ethylene imine) (PEI), a common polymer for industrial applications, as a cell transfection agent. PEI displays superior transfection efficiency to other polymer-based systems and is considered to be state-of-the-art polymer transfection reagent, available commercially under trade names such as JetPEI (PolyPlus Transfection, Inc.). PEI’s excellent transfection efficiency is attributed to its ability to effectively escape the endosome due to its proton sponge-like abilities of its backbone secondary amines, i.e., the capability of the molecule to act as a reservoir for protons. However, due to its cationic nature, PEI is cytotoxic.

While most research efforts focus on the modification of commercially-available polymers to improve nucleic acid delivery, several research groups have designed structurally-unique polymer transfection reagents (Figure 1-4), providing insight on the structural parameters that facilitate effective cell transfection and DNA expression. The design of synthetic polymers for effective and safe transfection can benefit from consideration of both macromolecular architecture and functionality. DNA binding affinity, ability to aid in endosomal escape, and biocompatibility are key features in transfection that can be addressed by synthetic vectors. The binding affinity of DNA to
cationic polymers is important as premature dissociation reduces cellular entry or leads to nucleic acid degradation in the extracellular environment or in the endosome.

Kataoka and coworkers have designed an array of polymeric transfection reagents over the past ten years with recent efforts based on functionalized PEG-block-poly(amino acid) structures (Figure 1-4).\textsuperscript{12,14} In one example, thiol moieties were incorporated to enable reversible crosslinking while controlling charge density.\textsuperscript{14} Such a system was designed to create stable crosslinked structures, encapsulating and protecting pDNA while circulating in vivo and later releasing DNA upon entry into the intracellular cytoplasm. This release is triggered by the presence of excess glutathione, a reducing agent found in large quantities in the cytoplasm as compared to other areas of the body. These studies found that the polymers yielding the highest protein expression balanced charge density and stability via disulfide crosslinking (\textit{i.e.}, if the degree of crosslinking was too high, decreased protein expression was observed). The structure of the most effective, environmentally-sensitive transfection reagent in these studies is shown in Figure 1-4. The crosslinking also improved the shelf-stability of these materials, which is important for commercial applications, and enabled the complexes to undergo freeze-thaw cycles without compromising their transfection abilities. However, these polyplexes only exhibited moderate protein expression and would need structural modifications to improve cell transfection and subsequent protein expression.

Kataoka \textit{et al.} also prepared polymers with similar backbones for the encapsulation and delivery of siRNA.\textsuperscript{12} As shown in Figure 1-4, the block copolymer incorporated both primary and secondary amines into the side chains to allow ionic complexation with the nucleic acid (primary amines) and to enhance the endosomal
buffering capacity (secondary amines). These polycations were found to be more effective than PEG-block-polylysine and comparable to RNAiFect, a commercial, lipid-based material from Qiagen, at knockdown of the targeted protein expression. However, in contrast to RNAiFect, these materials were stable in the presence of serum, showing no change in the subsequent knockdown efficiency, and still maintained greater than 75% cell viability.

![Chemical structures of various polymer-based transfection agents](image)

**Figure 1-4. Polymer-based transfection agents for siRNA and/or pDNA delivery**
Reineke, Liu, and coworkers have focused on creating new polymer transfection reagents by combining PEI-like segments with various biocompatible functionalities such as saccharide moieties. In an effort to combine the effective cell transfection and expression of PEI and the biocompatibility of chitosan, a new library of poly(glycoamidoamine) materials (Figure 1-4) was synthesized with varying length oligoamine segments and hydroxyl group stereochemistry. These polymers were able to maintain the biocompatibility as reflected in high cell viability typical of chitosan-based reagents while, in some cases, also achieving high cell transfection relative to PEI. It was also found that increased binding efficiency between the polycation and DNA, which was correlated to the length of the oligoamine moiety in this case, resulted in more effective cell transfection and DNA expression.

Building upon this work, the Reineke group recently used click chemistry to synthesize poly(glycoamidoamine)s with higher molecular weights than obtained by other condensation-based polymerization methods. Higher molecular weight polymers have been shown to create more stable polyplexes with DNA and therefore are desirable as polymeric transfection reagents. This synthetic methodology also allowed the introduction of triazole groups, which like pyrrole or imidizole-containing polymers, bind with DNA in a specific manner (via hydrogen bonding to the DNA base pairs) to increase complex stability. Other structural components were also included to enhance this transfection reagent including trehalose groups, which are known to enhance biocompatibility and prevent protein adsorption, and PEI-like oligoamine segments to promote electrostatic binding to DNA and aid in endosomal escape. The resulting polymers were found to be stable in water, serum, and salt, able to transfect cells in the
presence and absence of serum, and maintain high cell viability with respect to the commercial reagent JetPEI. By changing the polymer structure, it was found that the properties of polycation-DNA complexes were governed by a combination of polymer backbone stiffness, electrostatic interactions, and hydrogen bonding interactions. However, these polymers still did not achieve protein expression levels as found in lipid-based, commercial transfection reagents such as Invitrogen’s Lipofectamine 2000.

Our research efforts focused on preparing novel, tailored polymers for various biological applications based on a modular synthetic pathway. Amphiphilic graft copolymers were chosen for this research for their tunability and the ability to incorporate a wide range of functionality (e.g., oligopeptides, PEG, and reactive groups) into the backbone. This modularity enables control over factors such as charge density, hydrophilicity, and size, all of which are critical characteristics of polymers used in nucleic acid delivery.

1.5 Research Outlook

This thesis research focused on the syntheses of oligopeptide-functionalized polyesters and polyolefins (Figure 1-5). Polyester functionalization was geared towards applications in biomaterials, tissue engineering, and drug delivery by incorporating sequences that promote cell-adhesion (Chapter 2). These polyester-\textit{graft}-oligopeptide materials were prepared by a 1,3-Huisgen cycloaddition reaction, “click” chemistry, of an azide-terminated oligopeptide (prepared by Fmoc-based solid phase peptide synthesis (SPPS)) and alkyne-containing polyester (synthesized by ring-opening polymerization). Following the syntheses of these materials, they were analyzed by nuclear magnetic resonance (NMR) and organic gel permeation chromatography (GPC).
Figure 1-5. Oligopeptide-functionalized polyesters and polyolefins for biomaterials and nucleic acid delivery applications

The oligopeptide-functionalized polyolefins are described in **Chapters 3 and 4**. These polymers were designed for nucleic acid complexation, and therefore the oligopeptide sequences are intended to incorporate positively-charged moieties (e.g., oligolysine) for DNA and siRNA complexation. Due to the modularity of the synthetic procedure, these polymers will also be explored as a new general class of polyelectrolyte materials incorporating other charged amino acids into the polymer chain and observing their solution properties (**Chapter 3**). SPPS was used to prepare the requisite macromonomer, and ROMP was utilized to prepare these oligopeptide-functionalized polymers. These graft copolymers were then characterized ($^1\text{H}, ^{13}\text{C}$ NMR, organic and aqueous GPC) and studied to determine their unique solution properties (static and dynamic light scattering) and their potential as DNA delivery vectors.
1.6 References


(22) Fraser, C.; Grubbs, R. H. *Macromolecules* 1995, 28, 7248-7255.


CHAPTER 2
OLIGOPEPTIDE-FUNCTIONALIZED POLYESTERS

2.1 Functionalization of Polyesters

Incorporating functional groups into aliphatic polyesters represents a synthetic challenge due to the inherent hydrolytic sensitivity of esters. Commercially-available aliphatic polyesters are not suited for all desired polyester applications, thus numerous efforts have been made to modify their structure to enhance water-solubility and cell targeting.\textsuperscript{1-3} Conditions for polyester functionalization must be carefully chosen to prevent backbone degradation. Oligopeptide-incorporation into aliphatic polyester is particularly difficult, as state-of-the-art oligopeptide synthesis uses coupling and protection/deprotection conditions that are incompatible with the polyester backbone. Another approach is to conjugate a deprotected oligopeptide with a functionalized polyester. However, deprotection prior to coupling presents problems associated with oligopeptide functionality (\textit{i.e.}, amines, alcohols, and carboxylic acids) that can interfere with the intended grafting. In light of these problems, most attempts to decorate aliphatic polyesters with peptide sequences have involved end-group functionalization or use of functional initiators. To our knowledge, only one example of the synthesis of a polyester-\textit{graft}-oligopeptide was reported prior to this thesis research. This work was from Langer and coworkers who polymerized a cyclic dimer of L-lactic acid and protected L-lysine by ring-opening polymerization followed by deprotection of the primary amines (\textbf{Figure 2-1}).\textsuperscript{1} The C-terminus of an RGD-containing oligopeptide was then coupled to the pendent amine groups. Using this methodology, a small degree (less than 1 mole percent) of oligopeptide incorporation was achieved, but problems such as
crosslinking and backbone degradation were also encountered as would be expected from the reaction of the two multifunctional reagents.

![Figure 2-1. Synthesis of oligopeptide-functionalized polyesters by Langer and coworkers](image)

In order to address these issues, we wanted to examine whether the mild reaction conditions associated with the Huisgen 1,3-dipolar cycloaddition of azides and alkynes, an example of a “click” reaction, were applicable to aliphatic polyesters.4,5 Click-cycloaddition has recently gained popularity due to its high degree of specificity, functional group tolerance, and ability to proceed in either aqueous or organic solvents. This triazole formation is particularly attractive due to the ease with which these functional groups can be introduced into small molecules and polymers. Sharpless, Fokin, Finn, and coworkers have reported extensively on these reactions, and they along with others have applied click chemistry to small molecules,4,5 virus particles,6 cell membranes,7 dendrimers,8,9 and other polymers10 (Figure 2-2). In this research, the question of whether click chemistry could be applied to polyesters and enable the incorporation of oligopeptide sequences without polymer degradation was investigated.
Specifically, Cu(I)-catalyzed click chemistry was applied for grafting oligopeptides to alkyne-functionalized aliphatic polyesters. This method enables the “bio-tailoring” of aliphatic polyesters, structures already of interest in biomaterials research but potentially enhanced for tissue engineering and drug delivery by attaching oligopeptides.

**Figure 2-2. Synthesis and functionalization of materials using click chemistry**
2.2 Results and Discussion

Aliphatic polyesters containing pendent alkynes were prepared as shown in Figure 2-3 by Bryan Parrish (Emrick Research Group, Ph.D. 2006).\textsuperscript{11} α-Propargyl-δ-valerolactone 1, synthesized from δ-valerolactone and propargyl bromide, was then polymerized by Sn(OTf)_2-mediated ring-opening polymerization. The alkyne-containing lactone could be copolymerized with ε-caprolactone 2 or homopolymerized 3 in a controlled fashion, yielding aliphatic polyesters with molecular weights typically ranging from 6,000 – 16,000 g/mol with polydispersities between 1.1 – 1.2.

Due to its known cell binding properties,\textsuperscript{12,13} a RGD-containing sequence was selected as the oligopeptide target. In this research, an azide-functionalized oligopeptide 4 was prepared by Fmoc-based solid phase peptide synthesis (Figure 2-4), starting from a serine-loaded Wang resin and using the peptide coupling agent O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphosphate (HBTU).\textsuperscript{14} The oligopeptide sequence was end-capped with 6-bromohexanoic acid under carbo diimide coupling...
conditions (DIC, HOBt, DMF) and cleaved from the resin with a 95:2.5:2.5 mixture of TFA:water:triisopropylsilane (TIPS).\textsuperscript{15}

In the published synthesis\textsuperscript{11}, conversion of the oligopeptide-bromide to oligopeptide-azide was carried out following cleavage from the resin. This was achieved using excess sodium azide in DMSO at room temperature for three hours. The DMSO was removed, and the oligopeptide was purified by precipitation and characterized by NMR, IR, and mass spectrometry.

Some problems (\textit{e.g.}, residual DMSO) associated with this method led to a search for improved conditions. An on-resin azide functionalization was tried following the addition of 6-bromohexanoic acid and prior to resin cleavage. Excess NaN\textsubscript{3} in 85:15 DMF:MeOH was added to the resin, and the mixture was agitated for three hours. After this time period, it was found that the bromide to azide conversion went only to 75\% completion based on \textsuperscript{1}H NMR spectroscopy (Br $\alpha$-protons – triplet at 3.52 ppm, N\textsubscript{3} $\alpha$-protons – triplet at 3.32 ppm). Therefore, the resin was filtered, and fresh NaN\textsubscript{3} solution was added to the resin. After three hours of agitation, complete conversion to the desired azide group was observed by NMR. The SDGRG-N\textsubscript{3}\textsuperscript{5} sequence was then deprotected and cleaved from the resin with 95:2.5:2.5 TFA:water:TIPS and purified by precipitation in diethyl ether. The desired compound was isolated as a white solid in 99\% yield (based on resin loading) and characterized by \textsuperscript{1}H and \textsuperscript{13}C NMR, IR, and low and high resolution mass spectrometry, all of which supported the desired structure. High resolution mass spectrometry (HRMS-FAB) yielded a major [M+H]\textsuperscript{+} peak at 630.296, the exact value of the calculated mass, and the diagnostic azide stretch was observed by IR at 2097 cm\textsuperscript{-1}. 

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After synthesizing both the alkyne-containing polyesters 2 and 3 and azide-capped oligopeptide 5, the Cu(I)-catalyzed click reaction was used to prepare the desired oligopeptide-functionalized polyester (Figure 2-5). Initial attempts to synthesize aliphatic polyester-\textit{graft}-oligopeptides were carried out on the alkyne-functionalized homopolymer 3 to give 5 mole percent peptide grafting. To achieve this, compound 5 (0.05 eq) was dissolved in water, and an acetone solution of the alkyne-polyester
homopolymer 3 (1.0 eq) was added drop wise to the aqueous, rapidly stirring, peptide solution. Copper sulfate pentahydrate (0.4 eq) and sodium ascorbate (0.8 eq) were introduced into the solution, and the mixture was heated to 100 °C for three hours and stirred overnight at 80 °C. The solution was cooled to room temperature and diluted with an aqueous saturated NaCl solution. The product was extracted with methylene chloride, dried over magnesium sulfate, and concentrated on a rotary evaporator.

![Figure 2-5. Synthesis of oligopeptide-grafted polyester by click chemistry](image)

The peptide-functionalized polyester 6 obtained in this fashion was characterized by ¹H NMR spectroscopy and GPC (Figure 2-6). The success of the reaction was confirmed by the presence of signals in the ¹H NMR spectrum from δ 6.70-7.80 ppm, corresponding to the peptide amides and triazole. It should be noted that the azide-terminated SDGRG oligopeptide is not soluble in chloroform, and if physically mixed with the starting polyester, its proton signals were not observed in ¹H NMR spectroscopy in CDCl₃. The presence of signals in the amide region of the ¹H spectrum provides evidence for the covalent attachment of the oligopeptides to the polyester backbone.

To further support peptide grafting and investigate the integrity of the polyester backbone over time, GPC characterization in DMF was performed on the resulting
materials. GPC characterization of the peptide-functionalized product (Figure 2-6) changed only slightly from the starting material as expected for such a low grafting density. It is apparent from the GPC traces that no substantial crosslinking or degradation of the polyester occurred during the course of the reaction. Moreover, it appears that these polyesters are shelf stable. The weak signal at low retention time (high molecular weight) in the GPC trace is attributed to possible aggregation of the oligopeptide-functionalized aliphatic polyester in the eluent (DMF).

![GPC traces before and after click reaction](image)

**Figure 2-6.** GPC characterization of the starting polyester 3 (left) and the polyester-**graft**-oligopeptide 6 (right) after the click reaction

In summary, a new synthesis has been presented in which aliphatic polyesters are functionalized with oligopeptides. The synthesis relies on polymerization of alkynemodified lactones and subsequent grafting using click cycloaddition of azide-terminated oligopeptides. This approach represents a convenient, rapid, and versatile synthesis of functionalized aliphatic polyesters, in which the mild conditions associated with click chemistry allow for post-polymerization functionalization in the absence of polyester degradation. It is anticipated that the structural and functional variation
obtained by the methods outlined here will useful in several areas, including biomaterials and various encapsulation and delivery technologies.

2.3 Future Research

Future efforts on the optimization of the reaction conditions with respect to temperature and catalyst selection could provide valuable insight of the scope of this click methodology for polyesters. It was postulated that the high reaction temperature (100 ºC) needed for cycloaddition onto polyester backbone (many click reactions proceed rapidly at room temperature) was due to binding of the copper to the wide range of functionality in the oligopeptide side chains. In fact, binding of Cu(II) with peptide amides has been reported and may be the reason higher temperatures were required. Different copper-based catalyst systems have been developed for the optimization of click chemistry and involve the addition of various ligands and reducing agents. One useful system employs copper sulfate in conjunction with the ligand bathocuproinedisulphonic acid disodium salt hydrate and the reducing agent tris(2-carboxyethyl)phosphine hydrochloride. The system may reduce Cu(II)-peptide interactions due to ligand binding and facilitate the click reaction of the SDGRG sequence at lower temperatures.

Demonstrating versatility with respect to percent peptide incorporation and the oligopeptide sequence would also being interesting in further studies. Initial efforts focused on incorporating only five mole percent oligopeptide so as to maintain the organic solubility of the polymer and facilitate isolation and characterization. However, higher levels of oligopeptide may be desirable in certain applications. Also, demonstrating versatility of this methodology in terms of the selected oligopeptide
sequence is important. Many azide-terminated oligopeptides are in principle suitable for click chemistry. One proposed study would focus on clicking cationic oligopeptides to aliphatic polyesters for applications in nucleic acid encapsulation and delivery. While the polyolefin-\textit{graft}-pentalysine system offers a hydrolytically stable, robust backbone that can be used to encapsulate plasmid DNA as discussed in \textbf{Chapters 3-4}, a polyester-\textit{graft}-pentalysine (or other cationic amino acid) generates a polymeric encapsulation system that is degradable. Azide-functionalized pentalysine and pentaarginine sequences have been prepared by solid phase peptide synthesis as previously described for SDGRG. In a similar approach to the polyolefin system described in \textbf{Chapters 3-4}, variations in the polymer structure could be explored for such applications including the incorporation of other cationic sequences (lysine versus arginine) and PEG groups in conjunction with the peptide grafts.

\textbf{2.4 Experimental Section}

\textbf{2.4.1 Materials}

1-hydroxybenzotriazole (HOBt) (<5% water), piperidine (99+%), N,N'-diisopropylcarbodiimide (DIC) (99%), 2,2,2-trifluoroethanol (TFE) (99.5%), 6-bromohexanoic acid (98%), sodium azide (99.5%), TIPS (99%), and phenol (99+) were purchased from Sigma Aldrich (Saint Louis, MO). \textit{N},\textit{N}'-diisopropylethylamine (DIPEA) (99%) was obtained from Alfa Aesar (West Chester, PA). Copper (II) sulfate pentahydrate (ACS reagent grade) and TFA (reagent grade) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Fmoc-Gly-OH, Fmoc-Ser(But)-OH, Fmoc-Asp(OBu)-OH, Fmoc-Arg(Pbf)-OH, HBTU, and Fmoc-Ser(But)-loaded Wang resin
(100-200 mesh, loading density of 0.6 mmol/g) were purchased from Advanced ChemTech (Louisville, KY). Deuterated solvents CDCl₃ and D₂O were obtained from Cambridge Isotopes (Andover, MA). Sodium ascorbate (crystalline) was purchased from Source Naturals. CH₂Cl₂ was washed according to standard procedures and distilled over CaH₂. All other materials were used without additional purification.

2.4.2 Instrumentation

NMR spectra were recorded in CDCl₃, d₆-DMSO, or D₂O solutions using a Brüker DPX300, Brüker Avance400, or Brüker Avance600 spectrometer (w₁₃C = 0.25*w₁₁H). Molecular weights and polydispersity indices were measured by gel permeation chromatography in DMF relative to polystyrene standards (Scientific Polymer Products Mₛ= 503, 700, 1306, 2300, 4760, 12,400, 196,700 and 556,000 g/mol) on systems equipped with three-column sets (Polymer Laboratories 300 x 7.5 mm, 5 mm, 10⁻⁵, 10⁻⁴, and 10⁻³ Å pore sizes) and refractive-index detectors (HP 1047A) at room temperature (THF) and 50 °C (DMF) with a flow rate of 1 mL/min. High resolution mass spectral (HRMS) data were obtained on a JEOL JMS700 MStation. IR absorbance data was obtained on a Perkin Elmer Spectrum One FT-IR spectrometer equipped with a universal ATR sampling accessory.
2.4.3 Synthesis of α-Propargyl-δ-valerolactone (1)

\[ \text{O} \quad \text{O} \quad \text{O} \]

\( n \)-Butyllithium (42.5 mL, 93.5 mmol) was added by syringe to a solution of \( N,N \) -diisopropylamine (13.1 mL, 93.5 mmol) in 625 mL THF at -78 °C and stirred for 15 minutes. A solution of δ-valerolactone (8.51 g, 85.0 mmol) in 225 mL THF was added drop wise over 1.5 hours and then stirred for an additional 30 minutes. Propargyl bromide (11.4 mL, 102 mmol) and hexamethylphosphoramide (17.7 mL, 102 mmol) were added drop wise over 20 minutes. The reaction mixture was then warmed to approximately -30 °C, and the temperature was maintained while stirring for two hours. Excess aqueous ammonium chloride was added, and the reaction mixture was allowed to warm to room temperature. Volatiles were removed by rotary evaporation. The resulting product was dissolved in ether, washed with a saturated NaCl aqueous solution, diluted with hexanes, washed again with the NaCl solution, dried over MgSO₄, and concentrated. Column chromatography (gradient 0-30% EtOAc in hexanes) on silica gel followed by Kugelrohr distillation afforded 1 as a colorless, viscous liquid (8.68 g, 74% yield).

HRMS-EI (m/z): [M+H]+ calculated for \( \text{C}_8\text{H}_{11}\text{O}_2 \) 139.076, found 139.078. \(^1\)H NMR (CDCl₃, 300 MHz): \( \delta \) (CHCl₃ = 7.26 ppm) 4.28 (m, 2H, \( \text{CH}_2\text{O} \)), 2.62 (m, 2H, \( \text{COCH} \)), 2.46 (m, 1H, \( \text{COCH}_2 \)), 2.22 (sxt, \( J = 6.4 \text{ Hz} \), 1H, \( \text{CHCH}_2\text{CH}_2 \)), 1.97 (t-d, \( J = 2.7 \text{ Hz} \), \( J = 1.0 \text{ Hz} \), 1H, \( \text{C} \equiv \text{CH} \)), 1.89 (q, \( J = 6.3 \text{ Hz} \), 2H, \( \text{CH}_2\text{CH}_2\text{CH}_2 \)), 1.68 (m, 1H, \( \text{COCHCH}_2 \)) ppm. \(^{13}\)C NMR (CDCl₃, 75 MHz): \( \delta \) (CHCl₃ = 77.0 ppm) 172.8 (C=O), 81.1 (C=CH), 70.4 (CH₂O), 68.8 (CH=CH), 38.9 (CC=O), 24.1 (CH₂CH₂CH₂), 22.0 (CH₂CH₂), 20.7 (CH₂C=CH) ppm. IR(ATR): C≡C-H 3280, C=O 1724 cm⁻¹.
2.4.4 Representative Polymerization - Synthesis of (2)

Lactone 1 and ε-caprolactone were distilled over CaH$_2$. Glass reaction vessels were flame-dried three times under a stream of N$_2$(g). EtOH (110 mL, 1.7 M solution in THF) and Sn(OTf)$_2$ (150 mL, 3.7 x 10^{-2} M solution in THF) were introduced to the reaction vessel and allowed to stir for 15 minutes. Compound 1 (1.10 g, 8.0 mmol) and ε-caprolactone (0.91 g, 8.0 mmol) were then added to the vessel by syringe. The mixture was stirred at room temperature for 48 hours and then dissolved in acetone and precipitated into cold hexanes. Residual 1 was removed by passage through a plug of silica gel, eluting with 50:50 EtOAc:hexanes followed by elution of the product with acetone. Solvent was removed by rotary evaporation to give pure polymer 2 as a clear viscous liquid (1.83 g, 91%). GPC (THF): $M_n = 7.5 \times 10^3$ g/mol, PDI = 1.11. $^1$H NMR (CDCl$_3$, 300 MHz): δ (CHCl$_3$ = 7.26 ppm) 4.07 (m, 4H, CH$_2$O$_{PVL+CL}$), 2.58 (m, 1H, C=OCH$_{PVL}$), 2.45 (m, 2H, CH$_2$C=CH$_{PVL}$), 2.29 (tr, $J = 7.3$ Hz, 2H, C=OCH$_{2CL}$), 2.03 (m, 1H, C=CH$_{PVL}$), 1.64 (br m, 8H, CH$_2$CH$_2$CH$_2$O$_{PVL}$ + CH$_2$CH$_2$CH$_2$CH$_2$O$_{CL}$), 1.39 (br m, 2H, CH$_2$CH$_2$CH$_2$O$_{CL}$) ppm. $^{13}$C NMR (CDCl$_3$, 75 MHz): δ (CHCl$_3$ = 77.0 ppm) 174.1 (C=O$_{PVL}$), 173.6 (C=O$_{CL}$), 81.1 (C≡CH$_{PVL}$), 70.2 (C≡CH$_{PVL}$), 64.6 (CH$_2$O$_{PVL}$), 64.2 (CH$_2$O$_{CL}$), 44.0 (C=OCH$_{PVL}$), 34.2 (C=OCH$_{2CL}$), 28.4 (CH$_2$CH$_2$O$_{CL}$), 27.6 (CHCH$_2$PVL), 26.2 (CHCH$_2$CH$_2$PVL), 25.6 (C=OCH$_2$CH$_2$CH$_2$CL), 24.6 (C=OCH$_2$CH$_2$CL), 21.3 (CH$_2$C=CH$_{PVL}$) ppm. IR(ATR): C≡C-H 3283, C=O 1726 cm$^{-1}$. 

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2.4.5 Synthesis of Azide-terminated SDGRG (5) from Bromide-terminated SDGRG (4) using Method 1

The oligopeptide sequence SDGRG was synthesized according to standard Fmoc solid phase peptide synthesis using the batch wise process and the peptide coupling agent HBTU. Fmoc-Ser(But)-loaded Wang resin (3.12 g with loading density of 0.6 mmol/g) was weighed into an oven-dried glass-fritted reaction tube and swollen with 30 mL dry CH₂Cl₂ for 5-10 minutes. The Fmoc group at the N-terminus was cleaved by the addition of a 25/75 solution of piperidine/DMF (30 mL), followed by agitation with nitrogen for three minutes. The resin was filtered, and fresh piperidine/DMF (30 mL) was added. After agitating for 20 minutes, the resin was filtered and washed with DMF six times.

A solution of Fmoc-Asp(OBut)-OH (3.85 g, 9.35 mmol), HBTU (3.48 g, 9.17 mmol), and HOBut (1.26 g, 9.35 mmol) in 20 mL of anhydrous DMF was prepared. After the solution became homogeneous, DIPEA (3.28 mL, 18.70 mmol) was added, and the resulting mixture was added immediately to the resin. The resin was then agitated for one hour, filtered, and washed with DMF (three times). A 25/75 solution of piperidine/DMF (30 mL) was added, and the resin agitated for three minutes. After filtration, piperidine/DMF was again added to the resin followed by agitation for 20 minutes. The resin was then washed with DMF (six times). The above amino acid addition procedure was repeated for Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, and a second unit of Fmoc-Gly-OH.
Following the addition of the second Gly unit, a solution of 6-bromohexanoic acid (1.82 g, 9.35 mmol) and HOBt (1.26 g, 9.35 mmol) was prepared in 15 mL of dry DMF. DIC (1.45 mL, 9.35 mmol) was added drop wise to the solution and stirred for 20 minutes. The activated solution was added to the resin and agitated for one hour. After filtration, the resin was washed with DMF (six times) followed by CH₂Cl₂ (four times) to remove any residual DMF.

Two different approaches were then used to prepare the final azide-functionalized SDGRG₅. Initial efforts cleaved the peptide from the resin after this coupling reaction whereas latter studies streamlined the synthesis by introducing the azide-functionality prior to resin cleavage. To generate the material by the first method, the peptide was deprotected and cleaved from the resin by agitating with a 95/2.5/2.5 solution of TFA/H₂O/TIPS (30 mL) for three hours. The solution was filtered, and the cleavage procedure was repeated with 30 mL of fresh solution and 30 minutes of agitation. The resin was then washed with CH₂Cl₂ (three times), and the filtrate was concentrated by rotary evaporation, precipitated into diethyl ether, and stored at 4 ºC for several hours before filtration. The solid was isolated by filtration, rinsed with diethyl ether (three times), and dried under vacuum overnight to afford the Br-terminated oligopeptide 4 as a white powder in nearly quantitative yield (based upon the given resin-loading density).

HRMS-FAB (m/z): [M+H]⁺ calculated for C₂₃H₃₉N₈O₁₀Br 667.205, found 667.207. ¹H NMR (d₆-DMSO, 400 MHz): δ (DMSO = 2.50 ppm) 11.90 (br, 2H), 8.30 (br, 1H), 8.20 (d, J = 7.9 Hz, 1H), 8.07 (m, 2H), 7.88 (d, J = 7.7 Hz, 1H), 7.72 (br, 1H), 7.16 (br, 2H), 4.64 (m, 1H), 4.22 (m, 2H), 3.66 (m, 8 H), 3.50 (tr, J = 6.7 Hz, 2H), 3.07 (m, 2H), 2.13 (tr, J = 7.2 Hz, 2H), 1.78 (m, 2H), 1.64 (br, 2H), 1.50 (m, 6H), 1.35 (m, 2H) ppm. ¹³C
NMR (d$_6$-DMSO, 100 MHz): $\delta$ (DMSO = 39.52 ppm) 172.7, 172.0, 171.9, 170.7, 169.4, 168.7, 156.8, 61.4, 55.0, 52.3, 49.4, 42.1, 40.4, 36.4, 35.1, 35.0, 32.1, 29.1, 27.3, 24.9, 24.4 ppm. ATR-FTIR: O-H and N-H 3286.1, C-H 2937.0, C=O 1644.1, N-H 1531.9 cm$^{-1}$.

Bromide-terminated SDGRG 4 (1.17 g, 1.75 mmol) was dissolved in DMSO (3.5 mL, 0.5 M), and NaN$_3$ (0.13 g, 2.00 mmol) was added to the solution. The reaction was allowed to proceed for 12-18 hours at room temperature after which the solution was filtered through Celite. Following rotary evaporation and Kugelrohr distillation to remove the DMSO, the crude product was dissolved in a minimal amount of methanol, and the residual salts were removed by filtration. The remaining solution was precipitated from diethyl ether and filtered to afford the azide-terminated sequence 5 as a white powder (1.09 g, 99% yield). HRMS-FAB (m/z): [M+H]$^+$ calculated for C$_{23}$H$_{39}$N$_{11}$O$_{10}$ 630.296, found 630.296. $^1$H NMR (d$_6$-DMSO, 400 MHz): $\delta$ (DMSO = 2.50 ppm) 8.36 (br, 1H), 8.27 (m, 2H), 8.07 (m, 2H), 7.72 (d, $J = 7.4$ Hz, 1H), 7.23 (br, 2H), 4.58 (m, 1H), 4.23 (m, 1H), 4.14 (m, 1H), 3.59 (m, 8H), 3.29 (tr, $J = 6.8$ Hz, 2H), 3.07 (m, 2H), 2.13 (tr, $J = 7.3$ Hz, 2H), 1.76 (m, 2H), 1.50 (m, 6H), 1.28 (m, 2H) ppm. $^{13}$C NMR (d$_6$-DMSO, 100 MHz): $\delta$ (DMSO = 39.52 ppm) 173.4, 172.7, 172.6, 172.4, 170.6, 169.3, 168.8, 157.2, 61.8, 55.3, 52.4, 50.6, 49.7, 42.5, 42.0, 40.5, 36.8, 35.0, 29.4, 28.1, 25.8, 24.7, 24.5 ppm. ATR-FTIR: O-H and N-H 3261, N=N=N 2097, C=O 1645 cm$^{-1}$. 
2.4.6 Synthesis of Azide-terminated SDGRG (5) using Method 2

The second method for preparing the azide-functionalized SDGRG 5 followed the same methodology as the first approach until the resin cleavage. Following the addition of 6-bromohexamoic acid and prior to resin cleavage as previously described, a solution of NaN₃ (0.61 g, 9.35 mmol) in 85/15 DMF/MeOH was added to the resin and agitated for three hours. The solution was filtered, and a fresh NaN₃ solution was added to the resin and agitated for another three hours. After filtering the solution from the resin, the resin was washed with 85/15 DMF/MeOH several times to remove residual NaN₃ and then DCM to wash away the residual DMF. The oligopeptide was then cleaved by agitating the resin with 95/2.5/2.5 TFA/H₂O/TIPS (30 mL) for three hours. The filtrated was collected in a clean flask, and the resin was washed with fresh cleavage solution and DCM several times. The solution was concentrated on a rotary evaporator and dissolved in minimal MeOH. The oligopeptide was precipitated from diethyl ether and isolated by filtration. The resulting white solid was isolated in 99% yield. Characterization of the resulting materials agreed with the data previously collected by ¹H and ¹³C NMR, HRMS, and ATR-FTIR.
2.4.7 Click Reaction Conditions for Polyester-graft-SDGRG (6)

Azide-terminated SDGRG 5 (23 mg, 0.036 mmol) was dissolved in 2 mL of water and heated to 80 °C. The acetylene-functionalized homopolymer 3 (100 mg, 0.72 mmol acetylene) was dissolved in 1 mL of acetone and added drop wise to the rapidly stirring solution. Sodium ascorbate (122 mg, 0.61 mmol) and copper (II) sulfate pentahydrate (79 mg, 0.32 mmol) were then added, and the resulting dispersion was heated to 100 ºC for three hours and then lowered to 80 °C and allowed to proceed overnight. The reaction mixture was cooled to room temperature, diluted with a saturated NaCl aqueous solution, and extracted five times with CH2Cl2. The combined organic layers were then dried over MgSO4 and concentrated by rotary evaporation. The resulting product 6 was dried overnight under vacuum to yield a yellow viscous liquid in 50-70% yield. GPC (DMF, after 1 month): $M_n = 19,400$ g/mol, PDI = 1.07. $^1$H NMR (CDCl3, 600 MHz): $\delta$ (CHCl3 = 7.26 ppm) 6.70-7.80 (m, 6 H), 4.73-4.75 (m, 1 H), 4.30-4.32 (m, 2 H), 4.07-4.17 (m, 4 H), 3.64 (tr, $J = 6.4$ Hz, 2 H), 2.73 (m, 2 H), 2.38-2.60 (m, 6 H), 2.02 (tr, $J = 2.5$ Hz, 1
H), 1.64-1.78 (m, 8 H), 1.58 (m, 6 H) ppm. ATR-FTIR: C-H 2944, C-H 2864, C=O 1722 cm\(^{-1}\).

2.5 References

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CHAPTER 3

OLIGOPEPTIDE-FUNCTIONALIZED POLYOLEFINs: SYNTHESIS AND
CHARACTERIZATION OF A MODULAR CLASS OF POLYELECTROLYTES

3.1 Oligopeptide-functionalized Polyolefins as a Modular Class of Polyelectrolyte Materials

Polyelectrolytes display unique properties as a result of their macromolecular nature and electrochemical properties. The resulting properties of these materials along with their widespread applicability in areas such as gene therapy,1-3 tissue engineering,4,5 coatings,6 membranes,7,8 and oil recovery9,10 have drawn physicists, chemists, and biologists alike to study these materials. Polyelectrolytes display long-range interactions as a function of their charge density and charge strength, and other parameters such as backbone flexibility and molecular architecture (hydrogen bonding capability, hydrophobic forces) also play key roles in the resulting material properties.11

Despite extensive synthetic and characterization efforts, questions remain concerning the correlation between polymer architecture and the resulting properties. The lack of this fundamental knowledge is a result of the difficulties in preparing polyelectrolytes with well-defined architectures and parameters, which can be tuned to explore a range of polyelectrolyte properties. In response to this need, numerous research groups have pursued different avenues to achieve well-defined architectures and create systems with a large degree of modularity.

Conventional polyelectrolytes are typically vinyl polymers (i.e., based on CH$_2$=CH-X monomers) prepared by anionic12 and free-radical11,13 methods, where the
charge character is directly on the polymer backbone or on a pendent group. The negatively or positively-charged X-group is typically in the form of carboxylate, ammonium, sulfonate, or pyridinium moieties. The close proximity of pendent charged groups gives rise to steric and charge repulsion that present both synthetic and characterization challenges. As such, new syntheses that can simultaneously provide spacing of pendent, charged moieties and thus variable charge density would be valuable. Another challenge with such systems is that they result in different molecular shapes based upon the ionic strength of the surrounding environment. Therefore, by eliminating the shape dependence on ionic strength, rigid polymers can provide information that is difficult to interpret from vinyl-based systems. One example of the tunability of rigid-rod polyelectrolytes is provided by Wegner and coworkers where they synthesized sulfonated phenylenes and varied parameters such as charge density to determine their effect on the resulting material properties.

Building on the unique properties of polyelectrolytes, branched polyelectrolyte systems offer the ability to generate a range of structures, from linear polymers to soft nanoparticles that can be synthesized by controlling the degree of branching. In general, branched polyelectrolytes with well-defined molecular weights, branching degrees, and branching type are difficult to synthesize due to lack of functionality tolerance in many controlled polymerization techniques, making it challenging to elucidate the structure-property relationships in the resulting materials. Methods such as anionic, free-radical, macromonomer, and Suzuki coupling based techniques have been employed to synthesize such architectures and have utilized protection/deprotection strategies to incorporate various ionic groups. Due to its functional group tolerance,
metathesis chemistry and ROMP in particular enable the synthesis of wide variety of olefin-based polyelectrolytes with pendent carboxylate,\textsuperscript{14,21} ammonium,\textsuperscript{22} and oligopeptide\textsuperscript{23-25} groups. With the introduction of advanced ruthenium catalysts, ROMP enables the incorporation of carboxylic acids, alcohols, azides, and amides without the need for protecting groups and only requiring protection strategies for primary amines and unsaturation.

Herein, unique oligopeptide-functionalized polyelectrolytes based on a polyolefin backbone are introduced and offer control over parameters such as backbone rigidity, sign of the charged group, functional group incorporation, and molecular weight. These polyelectrolytes were synthesized on a multi-gram scale using Fmoc-based SPPS\textsuperscript{26} for monomer preparation followed by ROMP (\textbf{Figure 3-1}). In these studies, structure-property relationships were established by varying charge density (\textit{i.e.,} graft length and charge spacing) along the polymer backbone and characterizing solution properties by static and dynamic light scattering. The materials described here contain oligolysine and poly(ethylene glycol) (PEG) grafts, offering potential for applications in nucleic acid encapsulation and delivery.
3.2 Results and Discussion

3.2.1 Synthesis and Characterization of Lysine-containing Monomers

Cyclooctene monomers 7 and 8, containing pentalysine and lysine pendent groups, respectively, were prepared by Fmoc-based SPPS on a 2-chlorotrityl chloride resin with loading densities ranging from 1.0-1.4 mol/g. Before cleavage from the resin, the N-terminus of the peptide sequence was capped with 5-carboxylic acid-1-cyclooctene. The oligopeptide-substituted monomers were cleaved from the solid phase resin under mildly acidic conditions (4:1 dichloromethane:trifluoroethanol (DCM:TFE)), which enabled removal of the macromonomer from the resin without cleaving the protecting groups. Monomers 7-9 were characterized by $^1$H and $^{13}$C NMR spectroscopy, FTIR spectrometry, elemental analysis, mass spectrometry, and/or GPC.
In the $^1$H NMR spectra of 7-9, the cyclic olefin protons were seen at ~5.6 ppm. In addition, GPC traces of macromonomers 7 and 9 showed them to be monomodal and of very low polydispersity. Due to the affinity of amines for ruthenium, the Boc-protected forms of 7 and 8 were used for ROMP.

### 3.2.2 Synthesis and Characterization of Polycyclooctene-\textit{graft}-lysine 10

Cyclooctene-functionalized monomers 7-9 were polymerized by ROMP using Grubbs Generation III catalyst ($\{(H_2IMes)(3-Br-pyr)\}_{2}(Cl)_{2}Ru=CHPh\})^{30}$ to give the polymers shown in Figures 3-1 and 3-2 and characterized in Table 3-I. Initial studies focused on the homopolymerization of 5-pentalysine(Boc)-1-cyclooctene 7. Exploration of a variety of solvents and temperatures led to optimal polymerization conditions of 0.5 M of 1 in 10/90 DCM/TFE at room temperature for 40 minutes. Under these conditions, the Boc-protected polymer was obtained in greater than 80% conversion in 40 minutes and was then purified by precipitation from ether in 80-86% yield. The polymer was characterized by $^1$H and $^{13}$C NMR. The ring-opening of the cyclic olefin monomer was confirmed by the $^1$H NMR spectrum, which showed an upfield shift from 5.6 to 5.2 ppm in the olefin proton signals. GPC of this Boc-protected polymer was not feasible in THF or chloroform due to its limited solubility, and a low dn/dc with respect to DMF prevented reliable GPC characterization in this more polar eluent.

Polycyclooctene-\textit{graft}-pentalysine(Boc) was deprotected with 4 M HCl in 1,4-dioxane and methanol to give 10 (Figure 3-2) as a white powder in 95% yield after precipitation into acetone. Trace macromonomer was removed by centrifugation using a filter device (Millipore Amicon Ultra) with a molecular weight cutoff (MWCO) of 10,000 g/mol. 10 was then freeze-dried and characterized by $^1$H and $^{13}$C NMR, GPC,
and static and dynamic light scattering. GPC was performed in 0.5 M aqueous acetic acid with 0.3 M Na₂SO₄ using ultraviolet (UV) and refractive index (RI) detection. The GPC trace of 10 is shown in Figure 3-3, indicating the presence of polymer with monomodal molecular weight distribution and only trace amounts of macromonomer or cyclic oligomers. 10, prepared using a 50:1 ratio of Grubbs Generation III catalyst, had a GPC-estimated number-average molecular weight (Mₙ) of 87,000 g/mol and a polydispersity index (PDI) of 1.7 against PEG standards. Given the molecular weight overestimation expected from GPC on these graft copolymers, the molecular weight given by GPC is in reasonable agreement with theoretical values (for DP = 50, Mₙ of the chloride salt is approximately 49,000 g/mol). However, the solution properties of these densely-grafted polyelectrolytes in water make light scattering an important characterization technique as discussed in later sections.
Figure 3-3. Aqueous GPC chromatograms of deprotected 7 (dashed) and 10 (solid) in 0.5 M acetic acid, 0.3 M sodium sulfate aqueous buffer at a flow rate of 0.5 mL/min

Compound 2 was sufficiently soluble to carry out the polymerization at a higher concentration than 1 of 1.2 M in 50/50 DCM/methanol at room temperature. Boc-protected 11 was prepared using a 50:1 monomer:catalyst ratio with Grubbs Generation III catalyst, and the resulting polymer was amenable to characterization by GPC in THF. GPC relative to PEG standards on Boc-protected 11 gave $M_n$ and $M_w$ values of 7,500 and 11,000, respectively, with a PDI of 1.5. Using poly(methyl methacrylate) (PMMA) standards, these values were estimated at 13,000 ($M_n$) and 18,000 ($M_w$) with a PDI of 1.4. At a target DP of 50, the anticipated $M_n$ of 11 was approximately 16,000 g/mol.

Monomer 7 was copolymerized with PEG-functionalized macromonomer 9 ($M_n = 1,200$ g/mol, $M_w = 1,360$ g/mol, PDI = 1.09 relative to PEG standards according to THF GPC), which was prepared by anionic polymerization of ethylene oxide from 5-hydroxycyclooctene by Kurt Breitenkamp (Emrick Research Group, Ph.D. 2009). This ROMP copolymerization to synthesize 12 was catalyzed by Grubbs Generation III catalyst and proceeded to high conversion in seconds to minutes at ambient temperature.
In order to completely solubilize both monomers 7 and 9, the reaction was run at 0.6 M in 50/50 DCM/MeOH. Similar PEG-containing macromonomers are typically polymerized at higher concentrations to reduce cyclization, but due to the high percentage of solids in this polymerization, high reaction concentrations were not feasible. A 1:1 feed ratio of monomers 7 and 9 led to random copolymers composed of approximately 70:30 pentalysine:PEG grafts based on $^1$H NMR spectroscopy. Before deprotection of the acid-labile Boc group, these pentalysine and PEG-containing graft copolymers were also characterized by GPC in THF to reveal molecular weights in the range of the targeted DP of 50, which puts the theoretical $M_n$ around 60,000-65,000 g/mol depending upon the copolymer composition (PEG standards: $M_n = 36,000$, $M_w = 61,000$, PDI = 1.7 and PMMA standards: $M_n = 51,000$, $M_w = 75,000$, PDI = 1.5). At a DP of 50, with a 70:30 pentalysine:PEG ratio of the monomer units, the expected $M_n$ is about 63,000 g/mol.

Boc-protected 12 was deprotected according to the same procedure as 10 and 11 (4 M HCl in dioxane, methanol, three hours, room temperature). 12 was precipitated into diethyl ether, isolated in 93% yield as an off-white solid, and characterized by NMR spectroscopy and GPC. Dynamic and static light scattering were then used to analyze 12 in solution.
Table 3-I. Structure and characterization of 10, 11, and 12. All polymerizations were catalyzed by Grubbs Generation III catalyst using a 50:1 monomer:catalyst ratio.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Solvent</th>
<th>Time</th>
<th>$M_n$</th>
<th>$M_w$</th>
<th>$M_w$ (abs.)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.5 M 10/90 DCM/TFE</td>
<td>40 min.</td>
<td>87,000$^b$</td>
<td>144,000$^b$</td>
<td>48,000$^c$</td>
<td>1.7</td>
</tr>
<tr>
<td>11</td>
<td>1.2 M 50/50 DCM/MeOH</td>
<td>3 hrs.</td>
<td>7,500$^a$</td>
<td>11,000$^a$</td>
<td>36,000$^c$</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>0.6 M 50/50 DCM/MeOH</td>
<td>3 hrs.</td>
<td>36,000$^a$</td>
<td>61,000$^a$</td>
<td>200,000$^c$</td>
<td>1.7</td>
</tr>
</tbody>
</table>

a = Boc-protected analog analyzed by THF GPC relative to PEG standards
b = determined by aqueous GPC relative to PEG standards (0.5 M acetic acid, 0.3 M sodium sulfate)
c = absolute $M_w$ determined by static light scattering in 0.1 M NaCl aqueous solution (25°C)

3.2.3 Light Scattering Studies of 10, 11, and 12

Static and dynamic light scattering were used to determine the solution properties of 10-12 (Figure 3-2) as summarized in Table 3-II. Initial studies focused on the effect of salt concentration and pH on the solution behavior of 10 (Figure 3-4, Table 3-II).

Subsequent experiments focused on the effect of varying the graft length (1 versus 5 lysine groups) by comparing 11 with 10 (Figure 3-5, Table 3-II) and the effect of increasing charge spacing while maintaining the polymer hydrophilicity using 12 (Figure 3-5, Table 3-II).

3.2.3.1 Light Scattering Studies of 10: The Effect of Ionic Strength

Initial studies focused on the solution behavior of 10 with an emphasis on the effect of ionic strength and pH. The weight-average molecular weight ($M_w$), radius of gyration ($R_g$), and second virial coefficient ($A_2$) were determined by static light scattering (SLS) from the generation of Zimm plots for various conditions (Figure 3-4). While the
pK_a of lysine’s side chain amine varies based on its environment (as seen in proteins), it has a pK_a value of around 10 in its free amino acid form. As solutions in neutral water, the oligolysine grafts become protonated. If all the lysine groups release their chloride counterions when dissolved in water, the observed M_w of 48,000 correlates to a DP of around 60, in good agreement with the targeted DP of 50. Even after considering that some counterions will remain associated with the polymer due to counterion condensation, dispersion of individual polyelectrolyte molecules is seen in the concentration range studied. No aggregation of these highly charged, amphiphilic polymers was seen. The positive second viral coefficient (A_2) further indicated this polyelectrolyte to be in the good solvent regime. An R_g of 25 nm for 10 was measured in 0.1 M NaCl aqueous solution. This measured R_g is considerably larger than the R_g of a Gaussian chain, calculated to be 6 nm based on a monomer contour length of 1.2 nm and a degree of polymerization of 60 (based on polymer radius of gyration: Gaussian, R_g=(1/6)^{1/2}(Lp)^{1/2}; Rod-like, R_g=(1/12)^{1/2}L where L: chain contour length. lp: persistence length (=1.2nm)). At a fully-extended conformation, the estimated R_g would equal 23 nm. This experimentally-determined R_g of 10 indicates a significantly expanded conformation in low salt solution.

To investigate the effect of solution ionic strength on polymer conformation, z-average M_w, A_2, and R_g of 10 were measured in 0.5 M NaCl solutions. The decreased A_2 value of 10 observed in this environment indicated that the solvent quality became poorer at elevated ionic strength, and based on the Zimm plot, an M_w value comparable to what was found in low salt solution was observed at this high salt concentration. The slight discrepancy seen in the values of M_w at 0.1 M and 0.5 M NaCl can be attributed to the
standard practice of ignoring salt effects in Zimm plot analysis. The $R_g$ of 10 was significantly reduced from 25 nm in a 0.1 M NaCl solution to 15 nm in a 0.5 M NaCl solution.

**Figure 3-4.** Zimm plot of SLS data (left) and DLS (right) of 10 in 0.1 M aqueous NaCl at 25°C. Based on DLS, the inverse of relaxation time, $1/\tau$, as a function of the scattering vector squared, $q^2$, is plotted at several concentrations to determine the hydrodynamic radius $R_h$ of 10.

**Figure 3-4** shows the hydrodynamic radius ($R_h$) measured by dynamic light scattering for 10 in different polymer concentrations. The linear dependence of inverse relaxation time on $q^2$ is characteristic of a diffusive motion for 10 in water. It should be noted that in all of the 10 solutions studied, only one relaxation mode was observed. As shown in **Table 3-II**, $R_h$ changed very little (from 11 nm to 10 nm) in a 0.1 M versus 0.5 M NaCl aqueous salt solution, which contrasted the strong salt-dependence of $R_g$. This suggested that even though the size of 10 decreased significantly with increasing ionic strength, its hydrodynamic properties were relatively stable despite the solution changes. As a measure of chain extension, the $R_g/R_h$ ratio was also calculated at different salt concentrations. The large $R_g/R_h$ value (~2.3) in 0.1 M NaCl aqueous solution suggests a highly extended chain conformation, which collapses at higher salt concentration of 0.5
Table 3-II. Summary of static and dynamic light scattering studies of 10, 11, and 12

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conditions</th>
<th>M_w (g/mol)</th>
<th>R_g (nm)</th>
<th>R_h (nm)</th>
<th>A_2 ((mol·dm^3)/g^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.1 M NaCl*</td>
<td>48,000</td>
<td>25</td>
<td>11</td>
<td>1.6×10^6</td>
</tr>
<tr>
<td></td>
<td>0.5 M NaCl*</td>
<td>53,000</td>
<td>15</td>
<td>10</td>
<td>4.7×10^7</td>
</tr>
<tr>
<td></td>
<td>pH 2*</td>
<td>57,000</td>
<td>28</td>
<td>12</td>
<td>2.9×10^6</td>
</tr>
<tr>
<td></td>
<td>pH 12*</td>
<td>61,000</td>
<td>20</td>
<td>8.8</td>
<td>6.6×10^6</td>
</tr>
<tr>
<td>11</td>
<td>0.1 M NaCl*</td>
<td>36,000</td>
<td>27</td>
<td>6.5</td>
<td>1.3×10^6</td>
</tr>
<tr>
<td></td>
<td>0.5 M NaCl*</td>
<td>28,000</td>
<td>9.0</td>
<td>4.4</td>
<td>-4.2×10^6</td>
</tr>
<tr>
<td>12</td>
<td>0.1 M NaCl*</td>
<td>200,000</td>
<td>35</td>
<td>22</td>
<td>2.2×10^7</td>
</tr>
<tr>
<td></td>
<td>0.5 M NaCl*</td>
<td>220,000</td>
<td>39</td>
<td>23</td>
<td>2.7×10^7</td>
</tr>
</tbody>
</table>

* pH 7 + salt concentration = 0.1 M NaCl

M, as indicated by the lower R_g/R_h (~1.5). Large R_g/R_h ratios have also been observed for polymers with densely grafted neutral side chains, attributed to the molecules assuming extended conformations in solution. The extension of the chain backbone in a so-called molecular bottlebrush originates from a strong excluded volume interaction caused by crowding side chains, which pushes the otherwise flexible polymer backbone into an elongated conformation. Analogous to these bottlebrush molecules, 10, which contain pendent protonated lysines, exhibited an extended conformation due to the combined effects of steric and electrostatic repulsion of the oligopeptide side chains. This electrostatic repulsion was significantly screened when the solution ionic strength was increased from 0.1 to 0.5 M NaCl, as illustrated in the reduced R_g/R_h value in the 0.5 M NaCl solutions. It should also be noted that in high salt solutions, steric crowding alone from relatively short pendent lysine chains is not sufficient to support an extended polymer conformation. An extensive computational modeling study is currently being
undertaken to quantify the relative contributions of steric crowding and electrostatic interaction on conformational properties of polymers grafted with charged side chains.

3.2.3.2 Light Scattering Studies of 10: pH Effects

10 was seen to assume an extended conformation under conditions at which the grafts are largely protonated, due to a combination of steric crowding and electrostatic repulsion of the positively-charged grafts in 0.1 M NaCl solution. \( R_g \) and \( R_h \) of 10 were also measured at pH 2 and pH 12 (Table 3-II) (no added NaCl). At pH 2, 10 was observed to be slightly more extended than at pH 7, a result of lower salt screening effects. In the high pH environment, the number of protonated amines decreases, and the grafts become more flexible, resulting in a reduction of \( R_g \) and \( R_h \). Unscreened electrostatic repulsion among residual charges in the grafts is likely responsible for the conformation that is characterized by a high \( R_g/R_h \) ratio at 0.1 M NaCl.

3.2.3.3 Light Scattering Studies of 11

To further explore the effect of the pendent oligolysine grafts on solution properties, 11 was studied by static and dynamic light scattering in 0.1 M and 0.5 M NaCl aqueous solutions at neutral pH (Figure 3-5). The \( R_h \) of 11 was found to be extremely small (on the order of 6.5 and 4.4 nm for 0.1 M and 0.5 M NaCl solutions, respectively). The \( R_g \) of 11 in 0.1 M NaCl solution was found to be larger than would be expected for a globular polymer conformation. The molecular weight indicated individual polymer chains dispersed in water. Based on \( R_g/R_h \), 11 is viewed as exhibiting highly anisotropic conformation, which was even greater than the anisotropic behavior seen in 10. 11 was in a poor solvent regime at high salt concentrations as indicated by
the negative $A_2$ value measured in 0.5 M NaCl solution. Evaluation of these various parameters suggests that 11 behaves as a typical hydrophobic polyelectrolyte. Numerous studies\textsuperscript{37-41} have determined that when dissolved in water, strongly-charged hydrophobic polyelectrolytes adopt a “pearl-necklace” conformation in which the polymer chain consists of multiple mini-aggregates comprised of several monomer units, or “pearls,” connected by a string of uncollapsed monomers. Due to the repulsion between pearls, the pearl-necklace conformation becomes stiff. When the solution ionic strength was increased from 0.1 M to 0.5 M, the electrostatic repulsion among the pearls was screened, and the entire chain collapsed into a globular structure as evidenced by the decrease in $R_g$ and $R_h$. The values of $R_g$ and $R_h$ changed from 27 nm and 6.5 nm at 0.1 M to 9.0 nm and 4.4 nm at 0.5 M NaCl. Although these light scattering results for 11 are consistent with the pearl-necklace model, more direct observation of “pearls” is desirable. While a direct observation of pearls formed by a single homopolymer molecule continues to be a challenge, 11 appears to be an excellent candidate for future experimental verification of theoretical predictions.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sls_data.png}
\caption{Zimm plot of SLS data for 11 (left) and 12 (right) in 0.1 M aqueous NaCl at 25 °C}
\end{figure}
3.2.3.4 Light Scattering Studies of 12

Aqueous solutions of 12 were also studied by light scattering at both low and high ionic strengths to explore the effect of charge spacing by the introduction of non-ionic, hydrophilic moieties (Figure 3-5). Compared to 10, the $R_g$ and $R_h$ of the PEGylated copolymer were substantially higher. Given that the molecular mass of the PEG grafts are comparable to pentalysine and that the targeted DP of 12 is the same as 10, the expansion of 12 was unexpected. Incorporating non-ionic, hydrophilic PEG grafts was expected to reduce the strong electrostatic repulsion among the pentalysine moieties, which was shown to be responsible for the highly anisotropic conformation of 10. Therefore, based on the dilution of the electrostatic repulsion, 12 was expected to be smaller. However, the effective molecular weight of 12 based on light scattering was measured to be about three times larger than the value obtained by GPC. Furthermore, the copolymer conformation was stable to drastic changes in ionic strength, in contrast to the behavior of 10. From these observations, the copolymer chains likely formed aggregated structures composed of (on average) three chains. One possible origin of such aggregation could be the hydrophobic nature of the polyolefin backbone. The molecular origin of why such aggregates are comprised of only a finite number of these copolymer molecules remains to be fully investigated. The question of the dependence of the unimer-to-multimer transition on the PEG content is a fundamental problem in understanding the molecular origin of multimer formation.

3.3 Conclusions

Utilizing a combination of solid phase peptide synthesis and ring-opening metathesis polymerization, new polyelectrolyte materials were prepared, containing
hydrophobic backbones with pendent lysine and PEG grafts. These materials were easily prepared in multi-gram quantities, making them attractive candidates for material-based applications.

The solution behavior of these polyelectrolytes was tailored by changing the peptide graft length as well as the spacing of the charged moieties. Despite the flexible polycyclooctene backbone, polycyclooctene-\textit{graft}-pentalysine 10 exhibited an extended conformation in low ionic strength aqueous solutions due to combined influences of steric crowding and electrostatic repulsion. Screening of electrostatic repulsion at high ionic strength resulted in a more condensed structure. Pearl-like structures were found for the monolysine derivative 11 where the hydrophobic backbone collapsed on itself in the aqueous environment. However, with the introduction of only 30 mole percent PEG grafts, the resulting random copolymer 12 no longer adopted an extended conformation based on the \(R_g\) and \(R_h\) values in solution. Instead, small aggregates with a finite number of polymer molecules are formed. Various other structural parameters such as negative versus positive charge and PEG length are currently being studied to better understand the structure-property relationship of these polyelectrolytes and to explore their potential in nucleic acid delivery (for the cationic polyelectrolytes) and cartilage mimetics (in the case of negatively-charged derivatives).

### 3.4 Experimental Details

#### 3.4.1 Materials

Piperidine (reagent plus, 99%), 2,2,2-trifluoroethanol (TFE) (99.5%), diisopropylethylamine (biotech grade, 99.5%), methanol (anhydrous, 99.8%), 3-
bromopyridine (99%), and Grubbs Generation II catalyst ((1,3-Bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene) dichloro(phenylmethylene)(tricyclohexylphosphine)ruthenium, $\text{C}_{46}\text{H}_{65}\text{Cl}_{2}\text{N}_{2}\text{PRu}$) were purchased from Sigma Aldrich (Saint Louis, MO). $\text{N,N}$-Dimethylformamide (EM Science, guaranteed reagent, 99.8% and EM Science, anhydrous, 99.8%) and pentane (EM Science, ACS reagent) were bought from VWR International (West Chester, PA). Sodium chloride (reagent grade), HCl (concentrated), Millipore Amicon Ultra (MWCO 10,000) centrifugal filter devices, and Millipore hydrophilic PVDF membrane filters (0.22 mm diameter) were obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Fmoc-Lys(Boc)-OH, 1-hydroxybenzotriazole (HOBt) hydrate, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), and 2-chlorotryptyl chloride resin (1.4 mmol/g, 100-200 mesh) were purchased from Advanced ChemTech (Louisville, KY). Deuterated solvents d₆-DMSO and D₂O were obtained from Cambridge Isotopes (Andover, MA). CH₂Cl₂ was washed according to standard procedures⁴² and distilled over CaH₂. TFE was distilled over sodium bicarbonate.⁴² All other materials were used without additional purification.

### 3.4.2 Instrumentation

NMR spectroscopy was acquired on a Brüker Avance400 ($^1\text{H}$) and 100 ($^{13}\text{C}$) MHz NMR using a Brüker BBO5 probe. Mass spectrometry data were obtained on a JEOL JMS700 MStation high resolution two-sector mass spectrometer with low resolution electrospray ionization (ESI). Gel permeation chromatography (GPC) was performed in tetrahydrofuran (THF) and aqueous eluents. For analysis in THF, a KNAUER HPLC Pump K-501, UV Detector K-2600 (absorbance measured at 232 nm and 260 nm), and RI
Detector K-2301 were utilized and equipped with three PLgel 5 mm MIXED-D 300 x 7.5 mm columns (flow rate = 1.0 mL/min). Aqueous GPC was performed in 0.5 M acetic acid and 0.3 M sodium phosphate buffer with a flow rate of 0.5 mL/min. The aqueous system was comprised of a HP Series 1050 Pump, HP 1047A RI Detector, and Waters 486 Tunable Absorbance Detector (λ = 232 nm) utilizing three Waters Ultrahydrogel Linear Columns (mixed beads, 7.8 x 300 mm). Elemental analysis (CHN testing) was performed by Schwarzkopf Microanalytical Laboratory (Woodside, NY). Light scattering studies were performed with an ALV light scattering apparatus equipped with an ALV-5000 board. A green laser (COHERENT) with a wavelength of 514.5 nm was used as light source, and the temperature of the sample holder was held constant at 25±0.1 °C by a circulating water bath.

3.4.3 Synthesis of 5-pentalysine(Boc)-1-cyclooctene (7)

![Chemical Structure](image)

The pentalysine-functionalized macromonomer 7 was synthesized via Fmoc-based SPPS with the coupling agent HBTU using a 2-chlorotrityl chloride resin. To load the resin with the first amino acid unit, the 2-chlorotrityl chloride resin (5.00 g of resin with 1.40 mmol of functional groups per gram of resin, 7.00 mmol, 100-200 mesh) was weighed into an oven-dried, glass-fritted reaction tube and swollen with dry DCM (40 mL) for 5-10 minutes. The resin was filtered, and a solution of Fmoc-Lys(Boc)-OH
(6.56 g, 14.00 mmol), dry DIPEA (6.10 mL, 35.00 mmol), and dry DCM (30-40 mL) was added to the reaction tube. The tube was capped, vented, and agitated with nitrogen. After 30 minutes, the resin was filtered and rinsed with dry DMF three times. The remaining reactive groups on the resin were capped with a 80/15/5 dry DCM/methanol/DIPEA solution (40 mL, 2 x 10 minutes) and then rinsed with reagent grade DMF. The Fmoc group was then removed with a 25/75 piperidine/DMF cleavage solution (2 x 40 mL). The solution was added to the resin, and the resin was agitated for three minutes. Following filtration, fresh cleavage solution was added and agitated for 20 minutes. The resin was filtered and rinsed with DMF six times, DCM three times, isopropanol three times, and hexanes six times. The resin was dried by suction filtration for 15 minutes, transferred to a vial, and dried under vacuum for 24-36 hours. The loading density was estimated by the change in mass from the starting resin to the final resin. Loading densities for these syntheses were generally in the range of 0.7-1.4 mmol/g with lower loading densities targeted for longer oligopeptide sequences. To tailor the degree of functionalization of the resin surface, the number of equivalents of the amino acid added to the coupling solution was changed. When using 2.00 eq of amino acid as described above, loading densities generally ranged from 1.20-1.40 mmol per gram of resin.

Following the addition of the first amino acid residue, the dried resin (6.61 g, 9.25 mmol) was transferred to a dry, glass-fritted reaction tube and swollen with 40 mL of DCM for five minutes. Fmoc-Lys(Boc)-OH (21.68 g, 46.27 mmol), HOBt (7.09 g, 46.27 mmol), HBTU (17.20 g, 45.34 mmol), and DMF (60 mL, reagent grade) were combined in an oven-dried round bottom flask, and DIPEA (reagent grade, 16.12 mL, 92.54 mmol)
was added to form a clear, yellow solution. The activated amino acid solution was then added to the resin, and the resin was agitated for one hour, filtered, and washed with DMF three times. As previously described for the resin loading, the Fmoc protecting group was cleaved with 25/75 piperidine/DMF, after which the resin was washed with DMF six times. The same procedure was utilized to add lysine units until the desired oligopeptide sequence length was obtained.

Following the addition of the final lysine residue, the Fmoc protecting group was cleaved, and the resin was thoroughly washed with DMF. A solution of 5-carboxylic acid-1-cyclooctene (prepared in three steps from 1,5-cyclooctadiene\textsuperscript{27-29}, 7.14 g, 46.27 mmol), HOBT (7.09 g, 46.27 mmol), HBTU (17.20 g, 45.34 mmol), DIPEA (16.12 mL, 92.54 mmol), and 60 ml of DMF was then added to the resin and agitated from one hour. The resin was filtered and washed with DMF and DCM three times. The macromonomer was then cleaved from the resin under mildly acidic conditions (4:1 DCM:TFE). After 45 minutes, the resin was filtered into a clean flask, and fresh solution was added. The resin was agitated for another 45 minutes, filtered, and washed with 4:1 DCM:TFE and then DCM three times. This resulting filtrate was concentrated on a rotary evaporator and then precipitated into diethyl ether. After sitting at 4°C for several hours, the product was isolated by filtration and then dried under vacuum. The white, powdery product was obtained in 80-85% yield based upon the estimated loading density and was characterized by \textsuperscript{1}H and \textsuperscript{13}C NMR, ATR-FTIR, low and high resolution mass spectrometry, and elemental analysis. \textsuperscript{1}H NMR (d$_{6}$-DMSO, 400 MHz): δ (DMSO = 2.50 ppm) 12.49 (br, 1 H), 7.64-8.03 (br m, 5 H), 6.72 (br, 4 H), 6.40 (br, 1 H), 5.64 (br, 2 H), 4.04-4.30 (br m, 5 H), 2.86 (br, 10 H), 2.22-2.38 (br m, 2 H), 1.97-2.20 (br m, 3 H), 1.10-1.81 (m, 81 H)
ppm. $^{13}$C NMR (d$_6$-DMSO, 100 MHz): $\delta$ (DMSO = 39.52 ppm) 177.12, 177.08, 173.42, 171.99, 171.48, 171.41, 171.25, 155.51 (5 C), 129.97, 129.88, 77.31 (5 C), 52.70, 52.60, 52.39, 52.21, 51.81, 43.60, 40.35, 32.25, 31.98, 31.81, 31.67 (2 C), 31.22, 30.74, 29.89, 29.57, 29.25 (3 C), 29.14, 28.26 (15 C), 27.49, 27.35, 25.44, 25.37, 24.02, 22.53-22.86 (overlapping, 5 C) ppm. ATR-FTIR: 3282, 2931, 1683, 1630, 1521, 1452, 1392, 1365, 1249, 1169, 864 cm$^{-1}$. Low resolution ESI (m/z): [M+Na]$^+$ calculated for NaC$_{64}$H$_{114}$N$_{10}$O$_{17}$ 1317.83, found 1317.8. High resolution FAB (m/z): [M+Na]$^+$ calculated for NaC$_{64}$H$_{114}$N$_{10}$O$_{17}$ 1317.8261, found 1317.8959. Elemental analysis (CHN): calculated C = 59.33, H = 8.87, N = 10.81, found C = 58.28, H = 8.94, N = 10.44.

3.4.4 Synthesis of 5-monolysine(Boc)-1-cyclooctene (8)

2-chlorotrityl chloride resin was loaded with lysine as previously described. Following the loading of lysine on the resin, the resin was swollen, and a solution of 5-carboxylic acid-1-cyclooctene, HBTU, HOBt, DMF, and DIPEA was added. The resin was agitated for one hour, filtered, washed with DMF six times, and washed with DCM three times. 8 was cleaved from the resin as previously described for compound 7, and the resulting white solid was characterized by $^1$H and $^{13}$C NMR, ATR-FTIR, low and high resolution mass spectrometry, and elemental analysis. $^1$H NMR (d$_6$-DMSO, 400
MHz): δ (DMSO = 2.50 ppm) 12.39 (br, 1 H), 7.80-7.95 (br m, 1 H), 6.77 (br m, 1 H), 6.40 (br s), 5.64 (br m, 2 H), 4.05 (br m, 1 H), 2.86 (br m, 2 H), 2.21-2.38 (br m, 2 H), 1.93-2.21 (m, 3 H), 1.10-1.87 (br, 21 H) ppm. 13C NMR (d6-DMSO, 100 MHz): δ (DMSO = 39.52 ppm) 176.99, 176.91, 174.03, 174.02, 155.63, 130.02, 129.98, 129.95, 77.37, 51.69, 43.58, 43.49, 40.17, 39.96, 39.75, 39.55, 39.34, 39.13, 38.92, 34.26, 32.24, 32.08, 30.67, 29.88, 29.61, 29.17, 28.97, 28.32, 27.61, 27.42, 25.52, 25.46, 24.10, 24.04, 22.97 ppm. ATR-FTIR: 3314, 2929, 2861, 1689, 1646, 1524, 1451, 1392, 1366, 1249, 1166, 988, 861, 778, 712 cm⁻¹. Low resolution ESI (m/z): [M+H]⁺ calculated for C₂₀H₃₅N₂O₅ 383.26, found 383.3; [M+Na]⁺ calculated for NaC₂₀H₃₄N₂O₅ 405.24, found 405.2; [M+K]⁺ calculated for KC₂₀H₃₄N₂O₅ 421.35, found 421.3. High resolution FAB (m/z): [M+H]⁺ calculated for C₂₀H₃₅N₂O₅ 383.2546, found 383.2558. Elemental analysis (CHN): calculated C = 62.80, H = 8.96, N = 7.32, found C = 62.30, H = 9.02, N = 7.21.

3.4.5 Synthesis of PEG1200-functionalized Macromonomer (9)

In a flame dried air-free flask, 5-hydroxycyclooctene (6.8 mL of a 2.0 M solution in THF, 13.6 mmol) was added to dry THF (200 mL) while stirring under N₂. The solution was titrated with potassium napthalenide (0.2 M in THF) until a slight green end-point was observed (~ 70.0 mL). The cyclooctene alkoxide solution was stirred for an additional 30 minutes at room temperature before being cooled to 0 °C in an ice/water bath. Ethylene oxide (15.0 mL, 340.6 mmol) was condensed at -78 °C using a stainless
steel gas transfer manifold and then slowly warmed to room temperature while transferring to the cooled cyclooctene alkoxide solution under static vacuum. The reaction mixture was pressurized with argon, sealed, and stirred at room temperature for 16 hours. The solution was concentrated, and the residue was purified by column chromatography on silica gel (93/7 → 90/10 CHCl3/MeOH). The product recovered from the column was dissolved in a minimal amount of chloroform and precipitated into a hexane/diethyl ether mixture. A white powder was isolated by filtration and dried under vacuum to yield 12.5 g (83% yield) of PEG-functionalized macromonomer 9. 1H NMR (CDCl3, 400 MHz) δ (CHCl3 = 7.26 ppm) 5.62 (m, 2 H), 3.28-3.84 (complex, br m, 114 H), 2.54 (br s, 1 H), 1.28-2.36 (complex br m, 11 H) ppm. 13C NMR (CDCl3, 100 MHz) δ (CHCl3 = 77.16 ppm) 130.2, 129.6, 81.1, 72.7, 71.0, 70.7, 70.4, 67.8, 61.8, 34.2, 33.5, 25.9, 25.8, 22.8 ppm. ATR-FTIR: 3491, 2882, 1467, 1359, 1341, 1280, 1242, 1100, 1060, 959, 841, 725 cm⁻¹. GPC (THF, PEG standards): Mn = 1,200 g/mol, Mw = 1,360 g/mol, PDI = 1.09.

3.4.6 Synthesis of Polycyclooctene-graft-pentalysine(Boc) (10 protected).

Polycyclooctene-graft-pentalysine(Boc) (10 protected) was synthesized by ROMP of 7. Macromonomer 7 (0.88 g, 0.68 mmol) was slowly added to a stirring solution of dry TFE (1.22 mL). The solution was vortexed until complete dissolution.
Grubbs Generation III catalyst ([H2IMes)(3-Br-py)2(Cl)2Ru=CHPh]) was prepared according to the literature procedure.\textsuperscript{30} 12.0 mg of the catalyst (0.014 mmol, 50:1 monomer:catalyst ratio) was dissolved in dry DCM (136 mL) in a separate vial and immediately added to the macromonomer solution. The reaction (final concentration 0.5 M 10/90 DCM/TFE) was vortexed for 5-10 minutes, stirred under nitrogen for a total of 40 minutes, and quenched with ethyl vinyl ether (~1 mL). The reaction solution was precipitated into diethyl ether and isolated by filtration. After drying under vacuum, the light yellow polymer was obtained in 85\% yield and characterized by \textsuperscript{1}H and \textsuperscript{13}C NMR. \textsuperscript{1}H NMR (d\textsubscript{6}-DMSO, 400 MHz): \(\delta\) (DMSO = 2.50 ppm) 12.52 (br s, 1 H), 7.60-8.20 (br m, 5 H), 6.68 (br s, 4 H), 6.38 (br s, 1 H), 5.29 (br s, 2 H), 3.92-4.40 (br m, 5 H), 2.87 (br s, 10 H), 1.09-2.32 (complex br m, 86 H) ppm. \textsuperscript{13}C NMR (d\textsubscript{6}-DMSO, 100 MHz): \(\delta\) (DMSO = 39.52 ppm) 173.60, 171.25, 171.00, 155.53, 77.32 (5 C), 51.90-52.10 (overlapping, 2 C), 31.90, 30.80, 29.20, 28.27 (15 C), 22.40-22.60 (overlapping, 5 C) ppm. Polycyclooctene backbone carbons were not visible by \textsuperscript{13}C NMR in d\textsubscript{6}-DMSO.
3.4.7 Synthesis of Polycyclooctene-\textit{graft}-pentalysine (10)

Boc-protected 10 (0.512 g, 0.526 mmol) was dissolved in 5 mL of dry methanol, and excess 4 M HCl in 1,4-dioxane (2 mL) was added. The vented reaction was allowed to stir at ambient temperature for several hours. The reaction solution was concentrated and precipitated from acetone. The product was isolated by filtration, rinsed with acetone, and dried under vacuum overnight, yielding 93% (0.479 g, 0.369 mmol) of the desired product. Residual macromonomer was removed by centrifugation with filter devices. The purified polymer was freeze-dried, yielding a white porous solid, and analyzed by $^1$H and $^{13}$C NMR and aqueous GPC. $^1$H NMR (D$_2$O, 400 MHz): $\delta$ (H$_2$O = 4.79 ppm) 5.28-5.62 (br m, 2 H), 4.18-4.42 (m, 5 H), 2.88-3.10 (m, 10 H), 2.36 (br, 1 H), 1.23-1.94 (complex br m, 40 H) ppm. $^{13}$C NMR (D$_2$O + 2 drops d$_6$-DMSO, 100 MHz): $\delta$ (DMSO = 39.52 ppm) 180.88, 175.58, 175.35 (2 C), 175.12, 132.48 (2 C), 55.10, 55.02, 54.85, 54.49, 54.35, 40.76, 33.66, 32.14 (5 C), 31.30 (5 C), 27.89 (4 C), 27.79, 23.84, 23.66 (3 C), 23.57 ppm. Some polycyclooctene backbone carbons were not visible by $^{13}$C NMR in D$_2$O/d$_6$-DMSO. GPC (0.5 M acetic acid, 0.3 M Na$_2$SO$_4$ aqueous buffer): $M_n = 87,000$ g/mol, $M_w = 144,000$ g/mol, PDI = 1.7.
3.4.8 Synthesis of Polycyclooctene-\textit{graft}-monolysine(Boc) (11 protected)

Similar procedures were utilized to generate the monolysine derivative as used for the pentalysine analog. The polymerization was performed in 1.2 M 50/50 DCM/MeOH at room temperature for three hours and was catalyzed by Grubbs Generation III catalyst (50:1 monomer:catalyst). The resulting polymer was precipitated from diethyl ether, isolated in 89% yield, and characterized by $^1$H and $^{13}$C NMR and THF GPC. $^1$H NMR (d$_6$-DMSO, 400 MHz): $\delta$ (DMSO = 2.50 ppm) 12.39 (br s, 1 H), 8.72 (br s, 1 H), 7.29 (br m, 1 H), 6.37 (br s), 5.32 (br s, 2 H), 4.15 (br s, 1 H), 2.88 (br s, 2 H), 2.21 (br s, 1 H), 0.9-1.89 (complex br m, 25 H) ppm. $^{13}$C NMR (d$_6$-DMSO, 100 MHz): $\delta$ (DMSO = 39.52 ppm) 174.89, 173.87, 155.56, 129.91, 129.63, 77.33, 51.53, 45.06, 32.99, 32.68, 32.33, 32.15, 30.53, 30.06, 28.99, 28.46, 28.27, 26.81, 24.78, 22.85 ppm. GPC (THF, PEG Standards): $M_n = 7,500$ g/mol, $M_w = 11,000$, PDI = 1.5. GPC (THF, PMMA Standards): $M_n = 13,000$ g/mol, $M_w = 18,000$, PDI = 1.4.
3.4.9 Synthesis of Polycyclooctene-\textit{graft}-monolysine (11).

11 was deprotected according to the same procedure used for 10 and isolated as an off-white solid in 83% yield. $^1$H NMR (D$_2$O, 400 MHz): δ (H$_2$O = 4.79 ppm) 5.44 (br s, 2 H), 4.39 (br s, 1 H), 3.75 (br m, 3 H), 3.00 (br m, 2 H), 2.40 (br s, 1 H), 1.20-2.15 (complex br m, 16 H) ppm. $^{13}$C NMR (D$_2$O + 2 drops d$_6$-DMSO, 100 MHz): δ (DMSO = 39.52 ppm) 175.72, 131.82, 54.35, 54.02, 47.28, 40.74, 33.63, 31.45, 27.88, 23.99 ppm. Some polycyclooctene backbone carbons were not visible by $^{13}$C NMR in D$_2$O/d$_6$-DMSO.
3.4.10 Synthesis of Polycyclooctene-\textit{graft}-pentalysine(Boc)-\textit{co}-polycyclooctene-\textit{graft}-PEG (12 protected)

In a representative polymerization, 7 (0.52 g, 0.40 mmol) and 9 (0.49 g, 0.40 mmol) were copolymerized at room temperature with Grubbs Generation III catalyst (50:1 monomer:catalyst) in 0.6 M 50/50 DCM/MeOH. The reaction proceeded for three hours under nitrogen and was quenched with ethyl vinyl ether. The polymer was precipitated from diethyl ether, isolated by filtration, and freeze-dried. The Boc-protected polymer was isolated in 71% yield as an off-white solid. The resulting material was analyzed by $^1$H and $^{13}$C NMR and was determined to be composed of 70/30 mole percent pentalysine(Boc)/PEG1200. The relative molecular weight was estimated by GPC. $^1$H NMR (d$_6$-DMSO, 400 MHz): \( \delta \) (DMSO = 2.50 ppm) 12.49 (br s, 1 H), 7.65–8.14 (br m, 5 H), 6.69 (br s, 5 H), 6.38 (br s), 5.33 (br m, 4 H), 4.55 (br s, 2 H), 4.05-4.38 (br m, 5 H), 3.10-3.79 (complex br m, 114 H), 2.85 (br m, 10 H), 0.98-2.32 (complex br m, 98 H) ppm. $^{13}$C NMR (d$_6$-DMSO, 100 MHz): \( \delta \) (DMSO = 39.52 ppm) 173.42, 171.50, 171.20, 155.51, 129.94, 77.30 (5 C), 72.35, 70.15, 69.80, 67.51, 60.22, 52.22,
51.76, 31.83, 30.73, 29.14, 28.26 (15 C), 22.66 (5 C), 22.50 ppm. Some polycyclooctene backbone carbons were not visible by \(^{13}\)C NMR in \(d_6\)-DMSO. GPC (THF, PEG Standards): \(M_n = 36,000 \text{ g/mol}, M_w = 61,000, \text{PDI} = 1.7\).

### 3.4.11 Synthesis of Polycyclooctene-\textit{graft}-pentalysine-\textit{co}-polycyclooctene-\textit{graft}-PEG (12).

The Boc-protected copolymer (12 \textbf{protected}) was deprotected and purified using the same conditions as 10. The final product was an off-white solid and obtained in 93% yield. \(^1\)H NMR (D\(_2\)O, 400 MHz): \(\delta (H_2O = 4.79 \text{ ppm}) 5.44 \text{ (br s, 4 H)}, 4.38 \text{ (br s, 5 H)}, 3.08-3.20 \text{ (m, 2 H)}, 2.85-3.08 \text{ (m, 10 H)}, 1.18-2.52 \text{ (complex br m, 53 H) ppm.} \(^{13}\)C NMR (D\(_2\)O + 2 drops \(d_6\)-DMSO, 100 MHz): \(\delta \text{ (DMSO} = 39.52 \text{ ppm}) 175.67, 175.37 \text{ (2 C)}, 175.15, 73.32, 71.77, 71.15, 61.97, 55.16, 54.51, 40.80, 32.25, 31.37, 27.97, 27.87, 23.72, 23.61 \text{ ppm. GPC (0.5 M acetic acid + 0.3 M Na}_2\text{SO}_4\text{ aqueous buffer):} \ M_n = 45,000 \text{ g/mol}, M_w = 91,000, \text{PDI} = 2.0.\)
3.4.12 Light Scattering Studies: Solution Preparation

NaCl solutions with concentration of 0.1 M and 0.5 M were prepared by dissolving corresponding amounts of NaCl pellets in water purified by a MilliQ UF system (Millipore, Billerica, MA) with a resistance of 18.2 Ω. To adjust the pH of a solution, HCl (diluted from concentrated solution) was used. Stock solutions were prepared by dissolving the polymer in the NaCl solutions and were then allowed to equilibrate at room temperature for at least 24 hours prior to further dilution. Light scattering samples for each polymer were prepared at 3-4 concentrations, ranging from 0.78 to 5.85 g/L. 10 was analyzed at concentrations of 1.46, 2.93, 5.85 g/L (0.1 M NaCl aqueous solution) and 1.41, 2.83, and 5.65 g/L (0.5 M NaCl aqueous solution). At lower salt concentrations, 11 was characterized at solution concentrations of 0.78, 1.77, and 3.53 g/L and 1.45, 2.93, and 5.65 g/L for 0.5 M NaCl. Due to its high scattering abilities at low concentrations, 12 was studied by light scattering at four concentrations: 0.56, 1.12, 2.23, and 4.46 g/L (0.1 M NaCl) and 0.35, 0.80, 1.60, and 3.20 g/L (0.5 M NaCl).

3.4.13 Light Scattering Studies: Static and Dynamic Light Scattering

For static light scattering experiments, the scattering intensity of a toluene solution was first used as the standard. Sample solutions were directly filtered into pre-cleaned cuvettes by a syringe equipped with a 0.22 μm-diameter membrane filter. Static light scattering was carried out in the angle range of 35° to 135° with three readings at each angle. Dn/dc measurements were made on a refractometer and used to calculate absolute $M_w$ for 10 and 11. In 0.1 M NaCl, the dn/dc values of 10 and 11 were measured as 0.18 and 0.22. 12 aggregated in solution at the concentration range used to determine dn/dc, and therefore, its dn/dc in 0.1 M NaCl was estimated as 0.20.
In dynamic light scattering studies, the scattering intensity autocorrelation, \( g^2(t) \), was recorded and analyzed by CONTIN algorithm\(^4\) to generate a relaxation spectrum where a single dominant peak was identified. Its corresponding relaxation time, \( 1/t \), was then plotted against scattering vector square, \( q^2 \). The slope of a linear fit of \( 1/t \) vs. \( q^2 \) yielded the diffusion coefficient of 10 at that specific concentration, \( D(c) \). The diffusion coefficient of 10 at zero concentration, \( D_0 \), was obtained by extrapolating the \( D(c) \) vs. \( c \) curve to \( c = 0 \) with the relationship of \( D(c) = D_0(1 + kc) \) where \( k \) is a constant. To determine the hydrodynamic radius \( R_h \), the Stoke-Einstein equation, \( D_0 = k_BT/6\pi\eta R_h \), was applied where \( k_B \) is the Boltzmann constant, \( T \) represents temperature, and \( \eta \) is the solvent viscosity at \( T \).

### 3.5 Future Research

Additional research in the areas of synthesis and characterization can be envisioned to explore the modularity in this system. **Figure 3-6** illustrates additional macromonomers that have been synthesized and in some cases polymerized. Preparing these materials and analyzing the correlating solution properties could better explore the effects of backbone rigidity, cellular targeting groups, negatively-charged amino acids, different cationic amino acid moieties, and graft length. The final step in the illustrated design of these amphiphilic graft copolymers (see **Figure 4-2**) will be to functionalize the PEG end groups with targeting groups such as RGD or folic acid. Recent work in conjunction with Kurt Breitenkamp (Emrick Research Group, Ph.D. 2009) has resulted in the preparation of a PEG-RGD-functionalized macromonomer. Initial attempts at copolymerizing this macromonomer with other functionalized monomers were successful.
and offer promise for incorporating other biologically-relevant, oligopeptide sequences into these materials.

Figure 3-6. Oligopeptide-functionalized macromonomers prepared for future studies

3.6 References

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CHAPTER 4
DNA DELIVERY WITH OLIGOPEPTIDE-FUNCTIONALIZED POLYOLEFINS

4.1 Nucleic Acid Delivery

As effective delivery of nucleic acids is at the core of gene-based therapies, extensive research has been devoted to determining the effect of structural design on transfection. In polyplexes, DNA binding affinity, endosomal escape, and biocompatibility affect the transfection efficiency. The binding affinity of DNA to the transfection agent impacts its interactions with charged serum proteins, salts, anionic glycosaminoglycans on cell membrane in the extracellular matrix, and the highly acidic endosomal environment within the cell. Premature dissociation of the polyplex can result in reduced cellular entry and/or nucleic acid degradation extracellularly or in the endosome. Heterocyclic groups such as pyrrole and imidazole have been shown to strongly bind to DNA due to their ability to specifically hydrogen bond with DNA base pairs.1-4 Over the past 30 years, Peter Dervan and co-workers have studied these specific interactions between various polyamides based on pyrrole and imidazole groups and the minor groove of DNA in an effort to regulate gene expression and offer new therapeutic options for cancer and other diseases.5-8 Studies of specific interactions using pyrrole- and imidazole-based polyamides with the minor groove of DNA provide insight into polymer-DNA interactions and can aid in the development of cationic transfection reagents with optimal DNA binding affinity.5-9

These heterocyclic groups along with secondary amines as found in poly(ethylene imine) (PEI) also aid the polyplexes in escape from the endosomal compartments, one of
many barriers to nuclear delivery of DNA (Figure 4-1). After achieving cellular entry via pinocytosis, the polyplexes are trapped within endosomal compartments. The delivery vehicle must protect the encapsulated DNA due to the low pH found within these organelles as well as facilitate the polyplex’s release into the cytoplasm. Incorporating heterocyclic groups and secondary amines into the delivery vehicle buffer these endosomal compartments by absorbing protons generated by the endosomal proton pump, slowing the acidification of the organelle. This buffering by the delivery vector aids in the polyplex release from the endosome into the cytoplasm.10

Figure 4-1. Cellular barriers for DNA delivery to the nucleus

Adjusting electrostatic interactions between the polymer and DNA can increase polyplex stability. Researchers have demonstrated that the stability of the polyplex, and
subsequently the effectiveness of the transfection reagent, vary depending on whether lysine, ornithine, histidine, or arginine is the cationic moiety. While polylysine-DNA complexes perform transfection, they do so with very low efficiency relative to reagents such as linear and branched polyethylenimine PEI and polyamidoamine (PAMAM) dendrimers. The recent introduction of branched and dendritic polymers as transfection reagents provides insight into the role of macromolecular architecture in transfection efficiency and cytotoxicity. A systematic study of linear versus branched oligolysine and oligohistidine provides an example of the importance of both architecture and functionality in plasmid DNA and siRNA complexation and transfection. Branched polymers were found to give smaller polyplexes, while greater histidine-to-lysine ratios improved protein expression.

4.2 Oligopeptide-functionalized Polyolefins: System Design for Applications in Nucleic Acid Delivery

Polyolefin-graft-oligopeptide structures were prepared and tested experimentally for their utility in nucleic acid delivery. The concept, illustrated in Figure 4-2, consists of an amphiphilic graft copolymer containing a hydrophobic backbone with a hydrophilic, charged graft structure. The simplest amphiphilic graft system consists of a homopolymer with cationic grafts, which will enable the study of nucleic acid encapsulation resulting from the electrostatic interactions between a cationic polymer and negatively-charged plasmid DNA or RNA. PEG grafts, shown as the second example, are dispersed among the cationic grafts to enhance the biocompatibility and
protein-resistance of the encapsulated system. The PEG-corona, expected to form in water, will shield the encapsulated nucleic acid from enzymatic degradation in vivo and prevent protein adsorption during systemic circulation. In a more advanced system, the PEG grafts incorporated into the copolymer could be end-capped with targeting groups that may enable cell-specific delivery.

Utilization of the graft copolymer structure enables control of numerous system parameters that cannot be obtained from linear polymers including:

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**Legend**
- = Polyolefin or Polyester Backbone
++++++ = Oligonucleotide
--- = Poly(ethylene glycol)

= Targeting groups such as folic acid and RGD

**Figure 4-2. Design of oligopeptide-functionalized polyolefin materials for applications in nucleic acid delivery**
Control over Charge Density – By controlling the length of the cationic amino acid grafts, as well as the amount incorporated into the polymer backbone, the degree of excess cationic charge can be controlled. This may prevent the cytotoxicity levels seen in PEI, a current standard in polymeric, non-viral delivery systems.\textsuperscript{10}

Incorporation of Non-immunogenic, Protective Groups – PEG is frequently used in biomedical applications due to its biocompatibility and non-immunogenicity.\textsuperscript{16} By incorporating PEG grafts, a protective, non-immunogenic outer corona can protect the particle exterior of the encapsulated DNA and reduce non-specific interactions and protein adsorption during circulation.

Based upon these design principles, research focused on the preparation of hydrolytically stable polyolefins with cationic amino acid grafts via ROMP (Figure 4-3). The syntheses and characterization of these materials have been described already in Chapter 3. Nonionic, hydrophilic PEG grafts were incorporated into copolymer structures by copolymerization of PEG-functionalized cyclooctene macromonomers. To evaluate the role of architecture in transfection, these amphiphilic graft copolymers were compared with linear polylysine (PLL) and commercial transfection reagents with respect to protein expression and cell viability following transfection.

4.3 Results and Discussion

4.3.1 Materials

Amphiphilic graft copolymers, consisting of a hydrophobic polycyclooctene backbone decorated with pendent oligolysines, were evaluated for their ability to facilitate DNA transfection into cells. ROMP of Boc-protected lysine and PEG-
substituted cyclooctenes gave polymers 10 and 12 as shown in Figure 4-3. In polymer 10, prepared by ROMP of the Boc-protected 5-pentalysine-1-cyclooctene macromonomer 7, the pentalysine grafts are placed on average at every eighth backbone carbon atom. The salt of macromonomer 7 was used in transfection experiments for comparative purposes. Polymer 12 integrates PEG grafts into the structure by copolymerization with a PEG1200-substituted cyclooctene macromonomer to reduce the charge density but maintain water solubility. The solid phase peptide synthesis of macromonomer 7 and the syntheses of polymers 10 and 12 are described in detail in Chapter 3.  \(^{19}\)

Figure 4-3. Polymers used in polyplex formation and transfection
4.3.2 Complexation

Polymer 10 was first studied for its ability to complex plasmid DNA. Nuclease-free aqueous polymer solutions were added to DNA solutions at various N:P ratios (i.e., the ratio of protonatable nitrogens in the polymer (N) to DNA phosphates (P)). Thirty minutes after mixing, the solutions were analyzed by agarose gel electrophoresis (0.8 weight percent with ethidium bromide). For polymer 10, complete DNA complexation was obtained at N:P ≥ 2 as indicated by the absence of free DNA bands at these ratios as shown in Figure 4-4 (representative data for polymer 10e shown). Dynamic light scattering was used to evaluate the hydrodynamic size of the polymer-DNA complexes. As shown in Figure 4-4, polyplexes formed by complexation of plasmid DNA and polymer 10e are ~80 nm diameter particles with relatively low size distribution. These polyplexes were found to very stable over a 24-hour timeframe in water; no aggregation or size change was observed by light scattering.

Figure 4-4. Polyplex characterization: a) gel electrophoresis and b) dynamic light scattering of polymer 10e-DNA complexes
4.3.3 Cell Transfection Studies

Initial transfection experiments were performed with COS-1 cells obtained from the Schwartz Research Group (University of Massachusetts Department of Biology). Polymers 10 and 12 of the various molecular weights given in Figure 4-3 were complexed with the GFP-expressing reporter vector pZsGreen1-N1 and were analyzed 48 hours post-transfection using a plate reader, fluorescence microscope, and the CellTiter-Glo® Luminescent cell viability assay. For comparison, commercial transfection reagents jetPEI™ (PolyPlus Transfection), Lipofectamine™ 2000 (Invitrogen), and SuperFect (Qiagen) were also used for COS-1 transfection according to the optimized protocols supplied by the manufacturers.

Cells transfected with polyplexes containing polymers 10b-e at their optimal N:P ratios demonstrated substantial protein expression and typically higher cell viability than those treated with the commercial reagents (Figure 4-5). The degree of polymerization (DP) of 10 appeared to be important with increasing average DPs of 63, 75, 89, and 206 (polymers 10b, c, d, and e, respectively) providing better transfection efficiency than the lower molecular weight polymer 10a. It is believed that the stability of the polyplexes increases with increasing molecular weight, which results in greater protein expression as seen with these materials. Polyplexes using 10a (DP 30) gave very little protein expression as judged by fluorescence microscopy. In contrast, the highest molecular weight derivative polymer 10e (DP 206) showed greater overall gene expression than commercial transfection reagents jetPEI™ and SuperFect and lower expression than Lipofectamine™ 2000, while maintaining excellent cell viability. Increased cytotoxicity with higher molecular weight polymer derivatives was not observed as 10e at the optimal
N:P for transfection still maintained 99% cell viability in COS-1 cells. However, increased toxicity was observed at higher N:P as in the case of 10b. In order to obtain significant protein expression with this material, an N:P ratio of 4 was required but resulted in only 77% cell viability. Polymers 10b, c, and d also facilitated DNA transfection as indicated by protein expression levels but with lower effectiveness than 10e.

![Figure 4-5](image-url)

**Figure 4-5.** Relative fluorescence (left) and cell viability (right) of COS-1 cells treated with polymers 10a-e at their optimal N:P ratio for protein expression. Fluorescence was measured 48 hours post-transfection (plate reader filters $\lambda_{\text{exc}} = 485$ nm, $\lambda_{\text{em}} = 520$ nm).

When evaluated at comparable N:P ratios as polymer 10, pentalysine macromonomer 7 proved unsuitable for transfection (**Figure 4-6**). This is in agreement with reports by Szoka and coworkers on poor transfection performance with polyplexes formed from short oligolysine segments, which require substantially higher N:P ratios to effectively complex DNA.\(^\text{11}\) However, when 7 was polymerized to form amphiphilic graft copolymer 10, the resulting material behaved much differently, thus demonstrating the benefit of the multidentate interactions inherent to macromolecules and, in this case, the graft or comb-like structure designed to facilitate such interactions.
Linear PLLs, containing on average 126, 219, and 262 lysine repeat units, were also analyzed in COS-1 transfection experiments. Despite having a similar overall number of protonatable nitrogens as polymers 10a-c, which had on average 120, 254, and 301 protonatable nitrogens per polymer chain, these linear polymers gave low transfection efficiency. The highest molecular weight PLL (DP 262) showed the best transfection (Figure 4-6) but was still inferior to polymer 10 derivatives and the commercial reagents. The differing abilities of PLL versus polymer 10 as transfection reagents might be attributed to the role of macromolecular architecture (linear versus graft structure), as well as the amphiphilic nature of 10 with its hydrophobic backbone and hydrophilic oligolysine grafts. Amphiphilic molecules such as fusogenic peptides are known to preferentially segregate to cell membranes and aid in cellular entry.

The PEG-containing cationic graft copolymers of type 12 were found to be inferior transfection vehicles relative to 10 in accord with literature reports on PEGylated cationic polymer transfection reagents.20-23 This might be attributed to several factors including molecular weight (12 had an average DP of only 35) as well as poorer complexation due to a combination of increased steric hindrance and reduced charge density. Also, the presence of the PEG grafts may shield charge on the surface of the polyplexes, reducing cellular entry and protein expression. Although adding the PEG segments reduces the transfection in vitro, the incorporation of such groups would be advantageous in vivo for systemic delivery applications since PEG chains prevent non-specific protein adsorption. Therefore, future efforts should focus on evaluating higher molecular weight derivatives of polymer 12 and determining the optimal PEG-to-pentalysine graft ratio to maximize transfection efficiency for these structures.
Figure 4-6. Relative fluorescence (left) and cell viability (right) of COS-1 cells treated with polymer 10e, polymer 12, salt of macromonomer 7, linear PLL (DP 262), and commercial reagents jetPEI™, SuperFect, and Lipofectamine™ 2000. Fluorescence was measured 48 hours post-transfection (plate reader filters $\lambda_{\text{exc}} = 485$ nm, $\lambda_{\text{em}} = 520$ nm).

With HeLa cells, which are more difficult to transfec and more sensitive to toxicity than COS-1 cells, polymer 10e-based polyplexes competed favorably with all three commercial reagents. This is seen qualitatively in the fluorescence microscope images of the transfected cells in Figure 4-7. When polymer 10e served as the transfection reagent, little change in the cell morphology was observed, indicating minimal alteration to cell growth and minimal cytotoxicity. This contrasts the fluorescence images obtained in experiments with jetPEI™, SuperFect, and Lipofectamine™ 2000, in which significant morphological alterations and substantial cell death was observed. Figure 4-8 provides a quantitative comparison of 10e-containing polyplexes with commercial reagents in terms of GFP expression and cell viability. Even relative to Lipofectamine™ 2000, which out-performed all polymer 10 derivatives in the COS-1 experiments, 10e proved to be an excellent transfection reagent (relative fluorescence units for Lipofectamine™ 2000 = 12,000 versus 19,000 for polymer 10e) while maintaining high levels of cell viability (17% for Lipofectamine™ 2000 versus
92% for polymer 10e). Synthetic polymer delivery vectors are known to be less toxic than lipid-based and other systems, but they typically do not reach comparable protein expression levels. However, 10e was found to be extraordinarily effective in facilitating DNA delivery while still maintaining excellent cell viability. The appeal of amphiphilic graft copolymer 10e as a transfection reagent can be attributed to its balance of transfection efficiency and biocompatibility, a balance which becomes increasingly important in vitro for sensitive cell lines as well as in vivo for systemic delivery.

Figure 4-7. Fluorescence microscope images (λ_{exc} = 467-497 nm, λ_{em} = 516-556 nm) of HeLa cells transfected with complexes of pZsGreen1-N1 (GFP-expressing plasmid DNA) and various transfection reagents: polymer 10e (N:P 3), jetPEI™, Superfect, and Lipofectamine™ 2000.
Figure 4-8. Relative fluorescence (left) and cell viability (right) of HeLa cells transfected with polymer 10e (N:P 3) and commercial reagents jetPEI™, SuperFect, and Lipofectamine™ 2000. Fluorescence was measured relative to positive controls 48 hours post-transfection (plate reader filters $\lambda_{\text{exc}} = 485$ nm, $\lambda_{\text{em}} = 520$ nm). Deprotected macromonomer 5-pentalysine-1-cyclooctene 7, polymer 12, and polylysine were ineffective transfection reagents in HeLa cells (data not shown).

The experiments described indicate that the use of polymer 10e as a DNA delivery vector requires attention to multiple factors including the delivery vehicle’s molecular weight, amphiphilic nature, and architecture. Polymer molecular weight is known to influence transfection performance as seen for polymers 10a-10e and other reported systems. The high molecular weight of 10e also provides additional stabilization to the polyplexes, preventing premature DNA release and subsequent DNA degradation. Studies by Schaffer, et al. with PLL derivatives (DP 19, 36, and 180) demonstrate that intermediate molecular weight polymers are optimal delivery vehicles since they balance the need for polyplex stability with the requirement of eventual polyplex dissociation. In the case of high molecular weight PLL with a DP ~180, the DNA was unable to dissociate from the delivery vector, preventing eventual protein expression. However, in our studies with the molecular weight series for polymer 10, a
substantial increase in protein expression was observed with the highest molecular weight
derivative 10e (DP ~206). This implies that despite the large DP of 10e, which provides
stability for the polymer-DNA complex, other features of this graft copolymer facilitate
DNA release from the polyplex once it is within the cell. Intracellular trafficking studies
will be reported in the future to better elucidate this structure-property relationship.
Emrick Group Members Drs. Delphine Chan-Seng and Sangram Parelkar are currently
conducting these studies.

Moreover, we note that amphiphilicity is characteristic of naturally-occurring
fusogenic peptide segments, which are known to aid proteins in cellular entry and
endosomal escape by membrane interactions.29 Because 10e contains both hydrophobic
and hydrophilic domains, this polymer vector may enhance cellular entry and endosomal
escape in a manner similar to fusogenic peptides. In addition, the hydrophobic backbone
of 10e may interact with the hydrophobic domains of DNA, further stabilizing the
polyplex.

The comb-like architecture of 10e must also be considered when determining the
structural features which contribute to delivery efficiency. Pentalysine grafts are present
on average at every eighth carbon atom, and because of the close proximity of the lysines
within the graft and along the polymer backbone, it is likely that incomplete protonation
is achieved. Studies by Suh et al. with PEI, hydrophobically-modified PEI, and
polyallylamine show that the protonation of an amine in a multiamine-containing
compound suppresses additional protonation events.30 They also determined that
hydrophobic environments make protonation unfavorable. Therefore, we speculate that
in 10e the close proximity of the lysine groups, as well as that of the grafts to the
hydrophobic backbone, prevent complete protonation of the oligolysine chains at neutral pH, thus offering a buffering mechanism that might aid in endosomal escape in vitro.

4.4 Conclusions and Future Research

This chapter described a novel and effective transfection reagent based upon an amphiphilic, cationic, comb-like polyelectrolyte. These polyplexes effectively transfect COS-1 and HeLa cell lines, while maintaining high cell viability. These reagents are clearly competitive with commercial polymer-, dendrimer-, and lipid-based transfection reagents in terms of both protein expression and cell viability, and as such open possibilities for new transfection reagent choices among the biomaterials and gene delivery communities. To further elucidate the structure-property relationship of these materials, several future experiments could be envisioned with respect to oligolysine graft length, molecular weight, and copolymer composition. While pentalysine derivatives were evaluated, expanding the range of oligolysine graft lengths is important as a next set of experiments. While a range of graft copolymer molecular weights were investigated in these experiments, it is not yet known whether the optimal molecular weight polymers were used. Additional experiments to determine the ideal molecular weight for DNA delivery might lead to such optimization. Further investigation would also be beneficial in the area of copolymer composition. While initial investigations showed the polymer 2 offered significantly reduced transfection compared to polymer 1, it is important to incorporate PEG grafts for in vivo applications. Therefore, additional research exploring the role of molecular weight as well as copolymer composition (i.e., ratio of pentalysine:PEG grafts) on transfection efficiency may provide transfection reagents useful in in vivo applications.
4.5 Experimental

4.5.1 Materials

COS-1 (African green monkey kidney cells, *Cercopithecus aethiops*) and HeLa (human adenocarcinoma epithelial cervical cells) cell lines were purchased from American Type Cell Culture (ATCC, Manassas, VA) or donated by the Schwartz Research Group (University of Massachusetts, Department of Biology). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, sodium pyruvate, and Lipofectamine™ 2000 were purchased from Invitrogen Corporation (Carlsbad, CA). Agarose (molecular biology grade, low-EEO), ethidium bromide (1% solution, molecular biology grade), tris-acetate-EDTA (TAE) buffer (10x solution, electrophoresis grade), agarose gel-loading dye (6x, contains 15% Ficoll 400, molecular biology grade), Costar white-walled 96-well plates, Nalgene PES syringe filters (0.2 µm, 13 mm diameter), and low retention Eppendorf tubes were purchased from Thermo Fisher Scientific (Waltham, MA). Poly-L-lysine hydrobromide was obtained from Sigma-Aldrich (Saint Louis, MO). CellGro Eagle’s Minimum Essential Medium, trypsin with EDTA, and non-natural amino acid supplements were purchased from Mediatech, Inc. (Herndon, VA). Gel loading dye was purchased from Biorad (Hercules, CA). jetPEI™ was received from PolyPlus Transfection (San Marcos, CA). EndoFree Plasmid Maxi Kit and SuperFect Transfection Reagent were obtained from Qiagen (Valencia, CA). DH5α cells were donated by Jeff Kane and Professor Larry Schwartz (University of Massachusetts, Department of Biology). Nuclease-free water and CellTiter-Glo® Luminescent Cell Viability Assay were obtained from Promega Corporation (Madison, WI).
Prior to the encapsulation and cell transfection experiments, the reporter gene pZsGreen1-N1, a GFP-expressing plasmid with 4,700 base pairs, was purchased from Clontech (Mountain View, CA) and then amplified and purified. DH5α cells, *Escherichia coli* cells, which had been prepared for transformation, were used as hosts for bacterial transformation according to standard molecular biology procedures. The amplified DNA was isolated and purified with an EndoFree Plasmid Maxi Purification kit. UV analysis (Pharmacia Biotech GeneQuant II) was used to determine the concentration and purity (260/280 ratio) of the amplified DNA stock solution prepared by bacterial transformation.

**4.5.2 Gel Electrophoresis**

Agarose (0.48 g) was added to 1x TAE buffer (60 mL) to prepare a 0.8 weight percent agarose gel. The solution was heated until boiling and apparent dissolution. After cooling for five minutes, 3 μL of 1 weight percent ethidium bromide solution was added to the agarose solution. This mixture was poured into a gel cassette containing a 10-well comb and allowed to set for 30 minutes.

To prepare the polyplex solutions, 1 μg of DNA was diluted in nuclease-free water to obtain a final volume of 10 μL. Separately, polymer solutions of various concentrations were prepared. 10 μL of each polymer solution was added to the DNA solution and allowed to equilibrate for 30 minutes. 2 μL of nucleic acid loading buffer was mixed with the polyplex solution, and 18 μL of the final solution was loaded onto the gel. The gel was run using a FisherBiotech horizontal minigel (7 x 10 cm) setup and an Invitrogen PowerEase500 power supply at 60 volts for 60-90 minutes in 1x TAE buffer.
The gel was then imaged with a Spectroline Slimline UV transilluminator equipped with a Canon Powershot A620 camera.

### 4.5.3 Dynamic Light Scattering

Polyplexes were prepared in filtered, nuclease-free water in a black-walled 96-well plate with a total well volume of 100 μl each. Prior to analyses, the plate was centrifuged at 1,000 rotations per minute for 2 minutes to remove excess air bubbles. The plate was inserted into a Wyatt DynaPro Plate Reader equipped with a temperature control module (set to analyze at 37 °C) and allowed to equilibrate for 10 minutes. Acquisition time was set to 60 seconds, and three runs were performed in each well, which were averaged together. The wells were analyzed every six hours over a 24-hour period.

### 4.5.4 Cell Culturing

COS-1 and HeLa cells were cultured according to standard protocol\(^{32}\) and utilized for cell transfection experiments.

### 4.5.5 Cell Transfection

COS-1 and HeLa cells were cultured according to standard protocol, and low passage number cells (less than 20) were used for cell transfection experiments. COS-1 and HeLa cells were plated in white-walled 96-well plates at seeding densities of 7 x 10\(^3\) cells per well and incubated in 5% CO\(_2\) at 37 °C 24 hours prior to transfection. After 24 hours, the cells were 70-80% confluent and were transfected as described below.
The polymers and macromonomer (1-2 mg) were weighed into low retention Eppendorf tubes, dissolved in nuclease-free water, and sterilized by filtration. The stock solutions were diluted to the appropriate concentrations to enable complexation with DNA at various N:P ratios. DNA solutions (4x solution for each polymer concentration) were prepared in a 96-deep well plate. DNA (1 µg) was added to nuclease-free water (40 µL). 40 µL of the diluted polymer solutions were added to the DNA solutions and allowed to equilibrate for a minimum of 30 minutes. Prior to transfection, the cells were washed with serum-free media. The polyplexes were diluted with 120 µL serum-free media (DMEM for COS-1 cells, MEM for HeLa cells), and 60 µL of the diluted polyplex solution was added directly to the cells. Each condition was repeated in triplicate.

Commercial reagents PLL, jetPEI™, SuperFect, and Lipofectamine™ 2000 were also used as transfection reagents according to each manufacturer’s recommended protocol.

After 4 hours of incubation at 5% CO₂ and 37 ºC, 120 µL of serum-containing growth media (10% fetal bovine serum, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% non-natural amino acids) was added to each well. The cell media was replaced with 150 µL of fresh serum-containing growth media 24 hours after transfection, and the cells were analyzed after 48 hours by fluorescence microscopy (Olympus IX71 fluorescence inverted microscope equipped with an Olympus DP71 digital camera), by plate reader in fluorescence mode (BMG Labtech FLUOstar OPTIMA plate reader) and by the CellTiter-Glo® Luminescent Cell Viability Assay. Transfection data shown in Figures 4-5, 4-6, and 4-8 are averaged values obtained over multiple experiments. In each experiment, each unique condition (i.e., different polymer, N:P ratio, etc.) was tested in triplicate.
4.5.6 Cell Viability Assay

To determine cell viability 48 hours after transfection, Promega’s CellTiter-Glo® Luminescent Cell Viability Assay was performed according to the recommended commercial protocol. The CellTiter-Glo® buffer and substrate were equilibrated to room temperature, and the buffer was added to the substrate to form the CellTiter-Glo® reagent. After equilibrating the 96-well plate to room temperature, the cell media was replaced with 70 μL of serum-free media, and 70 μL of the mixed CellTiter-Glo® reagent was added to each well. The plate was mixed on an orbital shaker for 2 minutes and then allowed to sit for 10-20 minutes. Luminescence was recorded on a plate reader (BMG Labtech FLUOstar OPTIMA plate reader). The average luminescence values for each condition were divided by the average luminescence of the control cells (cells treated with media only) to determine the percent cell viability. As with the transfection data, cell viability results shown in Figures 4-5, 4-6, and 4-8 are averaged values obtained over multiple experiments. In each experiment, each unique condition (i.e., different polymer, N:P ratio, etc.) was tested in triplicate.

4.6 References

(1) Dervan, P. B.; Edelson, B. S. *Current Opinion In Structural Biology* 2003, 13, 284-299.
CHAPTER 5

FUTURE STUDIES

5.1 Oligopeptide-functionalized Polyesters

Click chemistry was successfully used to graft oligopeptide sequences onto hydrolytically degradable polyester backbones. To demonstrate the versatility of this technique, the conjugation of various other oligopeptide sequences to the polyester backbone could prove interesting. One proposed study would focus on using click chemistry to conjugate cationic oligopeptides to aliphatic polyesters for applications in nucleic acid delivery. While the polyolefin-\textit{graft}-pentalysine system described in Chapters 3-4 offers a hydrolytically stable, robust backbone that can be used to encapsulate plasmid DNA, a polyester-\textit{graft}-pentalysine (or other cationic amino acid) generates a polymeric encapsulation system that is degradable. Azide-functionalized tri- and pentalysine and pentaarginine sequences were prepared by solid phase peptide synthesis according to the same synthetic techniques previously described for SDGRG. Initial experiments were performed to determine if the trilysine-azide sequences could be clicked to the polyester backbone without significant degradation. Several reaction conditions were investigated, but additional experiments are necessary to determine whether click chemistry is a viable route for introducing cationic functionalities to polyesters. In a similar approach to the polyolefin system described in Chapters 3-4, variations in the polymer structure could be explored in future studies for such applications including the incorporation of other cationic sequences (lysine versus arginine) and PEG groups in conjunction with the peptide grafts.
5.2 Oligopeptide-functionalized Polyolefins as Novel Polyelectrolytes

To further explore these materials as novel polyelectrolytes, future synthetic efforts should focus on incorporating structural variations such as negatively-charged moieties and increased backbone rigidity. Along these lines, my synthetic efforts focused on varying the cyclic olefin and the charged moiety incorporated into the macromonomer. Norbornene-\textit{graft}-pentalsine(Boc) was prepared via SPPS by capping the \textit{N}-terminus of the oligopeptide sequence with commercially-available 5-norbornene-2-carboxylic acid. Cyclooctene-\textit{graft}-penta(aspartic acid) was synthesized with and without butyl(But)-protecting groups according to a similar procedure as 7 and standard Fmoc-SPPS techniques. The enhanced solubility contributed by the \textit{tert}-butyl protecting groups made the protected derivative easier to purify and polymerize and was used for preparing the negatively-charged polyelectrolyte materials. Upon GPC analysis of poly(cyclooctene-\textit{graft}-penta(aspartic acid)(OBut)), it was found that unlike the polymerization of 7, the aspartic acid-containing macromonomer had to be polymerized at a concentration of 1 M or above and at room temperature to prevent the formation of low molecular weight cyclics. When the polymerization was carried at elevated temperatures (40 °C and above) or at a concentration of 0.5 M, cyclics were observed in the GPC chromatogram. While both the norbornene- and aspartic acid-containing macromonomers were synthesized and polymerized, polymerization, deprotection, and purification conditions were not optimized, and the resulting polymers’ solution properties were not studied. Future efforts along these lines may provide valuable information on the range of polyelectrolytes that can be prepared in this class of materials.
Additional synthetic modifications can be incorporated to allow the PEG end groups to be functionalized with targeting groups such as folate and a RGD oligopeptide sequence. These groups have previously been incorporated into gene delivery systems to enable cell-specific interactions and selective cellular entry and can be incorporated as PEG end groups. Initial studies in this area were performed with Kurt Breitenkamp, Ph.D. (Emrick Research Group, Ph.D. 2009). An RGD-containing oligopeptide was prepared by SPPS and coupled with cyclooctene-PEG-carboxylic acid on the resin. The resulting compound was cleaved from the resin under mildly acid conditions, isolated, and characterized. Analyses by NMR, GPC, and MALDI support the synthesis of the desired cyclooctene-PEG-RGD macromonomer. Future synthetic efforts should focus on copolymerizing this macromonomer with other oligopeptide-functionalized macromonomers to create an array of materials with cell-targeting abilities.

5.3 Oligopeptide-functionalized Polyolefins for Nucleic Acid Delivery

To further explore the structure-property relationship of oligopeptide-functionalized polyolefins as nucleic acid delivery vectors, future experiments determining the role of oligolysine graft length, molecular weight, copolymer composition, the cationic group (e.g., lysine versus arginine), and incorporation of histidine groups to aid in endosomal escape may prove interesting. Ongoing efforts in the Emrick Research Group by Drs. Delphine Chan-Seng and Sangram Parelkar are focused on comparing the transfection efficiency and cytotoxicity of polycyclooctene derivatives containing 3, 4, and 5 lysine moieties per repeat unit as well as the determining the optimal molecular weight. The research described in this thesis showed preliminarily the effect of PEG moieties on the resulting copolymer’s transfection
properties. However, additional studies are needed to determine what the optimal cationic:PEG ratio is for both *in vitro* and *in vivo* applications.

Since the polyolefin materials were designed to encapsulate various nucleic acids, the application of these materials in siRNA delivery should also be explored. Preliminary experiments demonstrated successful siRNA encapsulation and *in vitro* gene knockdown with these oligopeptide-functionalized polyolefin delivery vectors. Due to the potential impact of siRNA therapeutics in treating such diseases as cancer and HIV, additional studies are warranted and may provide valuable insight on designing effective siRNA delivery vehicles.


